

REMARKS

Claims 1-68 are pending. The Examiner withdrew claims 51-67 from consideration. Claims 1-50 and 68 are under consideration.

In the present amendment, Applicants propose to cancel claim 19. Applicants propose to amend claim 1 to recite "wherein the fluorescent indicator is a nucleic acid binding molecule." That amendment is supported, for example, by original claim 19. Applicants propose to amend claim 26 to recite "wherein the first reaction composition and the second reaction composition are separate reaction compositions." That amendment is supported, for example, at page 14, paragraph 42. Applicants propose to amend claims 20-22 so that they depend from claim 3 and not from proposed canceled claim 19.

Applicants acknowledge the withdrawal of the rejection of claim 49 under 35 U.S.C. § 112, second paragraph.

I. Rejection of Claims 1-25 and 68 Under 35 U.S.C. § 103(a)

The Examiner maintained the rejection of claims 1-25 and 68 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Pritham et al. (J. Clinical Ligand Assay (1998) 4:404-412), in view of Johnston-Dow et al. (U.S. Patent No. 6,103,465). Specifically, the Examiner alleges that the applied art would have provided a reasonable expectation of success because "the reference of Johnston-Dow indicates that sequencing was performed with no purification of the PCR product using the TaqCS polymerase enzyme. . . ." Final Office Action at page 3.

To establish a *prima facie* case of obviousness, it is the Examiner's burden to show that there would have been a reasonable expectation of success. See MPEP § 2142 at 2100-128 (8th ed. rev. 2, May 2004) ("The examiner bears the initial burden of factually supporting any *prima facie* conclusion of obviousness."). A reasonable expectation of success requires "at least some degree of predictability." MPEP § 2143.02 at 2100-133. "Evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness." *Id.*

Claims 1 and 68 recite "directly sequencing the at least one amplification product." Proposed amended claim 1 recites that the "at least one amplification product" is present in a "reaction composition comprising a fluorescent indicator, wherein the fluorescent indicator is a nucleic acid binding molecule." Claim 68 recites that the "at least one amplification product" is present in a "reaction composition comprising an intercalating fluorescent indicator." An "intercalating fluorescent indicator" is a nonlimiting example of a nucleic acid binding molecule. See specification at page 8, paragraph 23.

It would have been unpredictable at the time the invention was made whether an amplification product could be directly sequenced in the presence of a fluorescent indicator that is "a nucleic acid binding molecule" or in the presence of "an intercalating fluorescent indicator." For example, it would have been expected that a fluorescent nucleic acid binding molecule or an intercalating fluorescent indicator might interfere with any one or more of the following: primer annealing and/or polymerization from a nucleic acid template during a sequencing reaction; the electrophoretic mobility of products generated by the sequencing reaction; and/or the detection of products

generated by the sequencing reaction. DNA sequencing is generally known to be highly sensitive to factors such as template purity and the presence of contaminants in the sequencing reaction. See, e.g., enclosed documents by Ausubel et al. Current Protocols in Molecular Biology: (Supp. 21 1993), at pages 7.4.26-7.4.35 (e.g., at 7.4.26, col. 2, under “Troubleshooting”); and Sambrook et al., Molecular Cloning: A Laboratory Manual (3rd ed. 2001), pages 12.56-12.59 (e.g., col. 2, rows 2 and 7 of Table 12-13) and pages 12.94-12.100 (e.g., at 12.98, second to last paragraph: “. . . it is critical to use template DNA clean and free of any contaminants that might interfere with primer annealing [in automated fluorescence-based DNA sequencing reactions]”).

Thus, directly sequencing the amplification product in the presence of a fluorescent indicator that binds nucleic acid might have been “obvious to try.” However, the Examiner has failed to establish that one of ordinary skill in the art would have had a reasonable expectation of success in carrying out such a method. See *Amgen Inc. v. Chugai Pharmaceutical Co.*, 18 USPQ2d 1016, 1022, 23 (Fed. Cir. 1991) (affirming the district court’s finding of no reasonable expectation of success on the grounds that “[w]hile . . . it might have been feasible, perhaps obvious to try, to successfully probe a [genomic DNA library], it does not indicate that the [claimed] gene could have been identified and isolated with a reasonable likelihood of success”) (emphasis added). (A copy of that case is enclosed.)

That there would have been no reasonable expectation of success is further suggested by the Applicants’ own disclosure. As discussed in the specification, the Applicants performed experiments to specifically explore the possibility that a fluorescent nucleic acid binding molecule would interfere with DNA sequencing. See

specification, e.g., at page 19, paragraph 57. The Applicants reported that “[t]he sequenced reaction product that was subjected to the low resolution HLA-DRB typing, containing SYBR® Green I, was compared to a sequencing reaction product of the same sample and amplification product containing no SYBR® Green I. The results show no detectable adverse effect on the sequencing reaction by the fluorescent indicator.” *Id.*; see also Figure 3. Clearly, the Applicants contemplated that SYBR® Green I might have an “adverse effect on the sequencing reaction” or else they would not have compared sequencing reactions performed in the absence SYBR® Green I with sequencing reactions performed in the presence of SYBR® Green I.

Indeed, it is the Applicants’ own work that established a reasonable expectation of success with direct sequencing of an amplification product in the presence of a fluorescent nucleic acid binding molecule. The Applicants’ own work, however, cannot be used to establish a reasonable expectation of success. See *In re Vaeck*, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991) (“[T]he reasonable expectation of success must be founded in the prior art, not in the applicant’s disclosure.”); see also *Life Techs. v. Clontech Labs.*, 56 USPQ2d 1186, 1191 (Fed. Cir. 2000) (“That the inventors were ultimately successful is irrelevant to whether one of ordinary skill in the art, at the time the invention was made, would have reasonably expected success.”). (Copies of those cases are enclosed.) Indeed, it is the Examiner’s burden to establish that the cited art would have provided one of ordinary skill in the art with a reasonable expectation of success. See MPEP § 2142 at 2100-128.

Because there would have been no reasonable expectation of success, claims 1 and 68 would not have been obvious over Pritham in view of Johnston-Dow. Claims 2-

25 ultimately depend from claim 1. Thus, those claims also would not have been obvious over Pritham in view of Johnston-Dow. Applicants respectfully request withdrawal of the rejection of claims 1-25 and 68 under 35 U.S.C. § 103(a).

II. Rejection of Claims 26-50 Under 35 U.S.C. § 103(a)

The Examiner maintained the rejection of claims 26-50 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Pritham in view of Johnston-Dow and in further view of Wittwer et al. (U.S. Patent No. 6,174,670). Specifically, the Examiner alleges that “the language ‘combining nucleic acid from the sample with at least one set of reaction composition comprising a first reaction composition and second reaction composition, . . .’ can be interpreted as that the nucleic acid is combined with two compositions in one reaction or the nucleic acid is combined with first composition in one reaction and the nucleic acid is combined with second composition in another reaction.” Final Office Action at page 4.

Although Applicants assert that the Examiner’s interpretation of the claim language is not reasonable for the reasons of record in the Amendment and Response filed September 15, 2004, Applicants propose to amend claim 26 to recite “wherein the first reaction composition and the second reaction composition are separate reaction compositions” solely to expedite prosecution.

To establish a *prima facie* case of obviousness, the Examiner must show that the cited documents would have taught or suggested each and every element of the claims. MPEP § 2143 at 2100-129. The Examiner has already conceded that “[t]he teachings of Pritham et al. and Johnston-Dow et al. do not indicate that there are two reaction compositions involved in the methods.” See Office Action mailed June 15, 2004, at page 4.

Wittwer would have failed to cure the deficiencies of Pritham and Johnston-Dow. In the passage of Wittwer cited by the Examiner (col. 13, line 62 through col. 14, line 29), for example, Wittwer discusses a method that uses two pairs of oligonucleotides, one pair specific for a selected template and one pair specific for a positive control template, in a *single* reaction mixture. See col. 13, line 65, and col. 14, line 15. Indeed, Wittwer does not at any point discuss combining nucleic acid from a sample with a first reaction composition and a second reaction composition that are “separate reaction compositions.” Wittwer also does not discuss “determining whether the at least one amplification product is present in both the first reaction composition and the second reaction composition from the intensity of signal from the fluorescent indicator in the second reaction composition” and “determining the sequence of the at least one amplification product of the first reaction composition if the at least one amplification product is present in the first reaction.” Thus, the cited documents, either singly or in combination, would have failed to teach or suggest all the elements of claim 26.

The Examiner further states that “[r]egardless how the claim language is interpreted, the teachings of Wittwer et al. still read on the limitations of the claims (See column 13, lines 63-76 and column 14, lines 1-43).” Office Action at page 5 (emphasis added). Thus, the Examiner appears to argue that, even if the instant claims are interpreted as reciting “separate” reaction compositions, Pritham and Johnston-Dow in view of Wittwer would have taught or suggested all the claim elements.

Applicants respectfully disagree with the Examiner. The Examiner has provided no explanation as to how Wittwer, either singly or in combination with Pritham and Johnston-Dow, would have taught or suggested all the claim elements, assuming that

the instant claims are interpreted to recite "separate" reaction compositions. Indeed, neither the cited portion of Wittwer nor any other portion of Wittwer, either alone or in combination with Pritham and Johnston-Dow, would have taught or suggested all the claim elements for the reasons discussed above. Thus, the Examiner is incorrect in arguing that Pritham and Johnston-Dow in view of Wittwer would have taught or suggested all the claim elements "regardless how the claim language is interpreted."

Because the cited documents, either singly or in combination, would have failed to teach or suggest all the elements of claim 26, that claim would not have been obvious over Pritham in view of Johnston-Dow and in further view of Wittwer. Claims 27-50 ultimately depend from claim 26. Thus, those claims also would not have been obvious over Pritham in view of Johnston-Dow and in further view of Wittwer. Withdrawal of the rejection of claims 26-50 under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

Applicants respectfully request the reconsideration and reexamination of this application and the timely allowance of the application. In the event that the Examiner does not find the claims allowable, Applicants request that the Examiner contact the undersigned at (650) 849-6778 to set up an interview.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

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DNA SEQUENCING

7

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tides from the primer are of interest, four factors can be varied, so as to produce a greater percentage of terminations in this region: (1) the concentration of the three dNTPs in the short labeling mix can be reduced (e.g., 3- to 5-fold); (2) the time of the labeling reaction can be reduced (e.g., to 2 min); (3) the dNTP/ddNTP ratio in the termination mixes can be decreased (e.g., ~5-fold) by raising the ddNTP concentration; and (4) the DNA concentration can be increased (e.g., 2-fold). It is not necessary to vary all of these factors; in most cases the size range of sequencing products can be closely regulated by varying just the concentration of the three dNTPs in the labeling reaction.

If the sequence just after the primer is the major region of interest, the intensity of the bands corresponding to this region can be increased by the addition of Mn^{++} to the labeling/termination reactions (alternate protocol; Tabor and Richardson, 1989b, 1990). Use of Mn^{++} reduces the discrimination against ddNTPs by Sequenase by ~4-fold and thus has the same effect as increasing the ddNTP to dNTP ratio by a factor of four. As a consequence, the average extension of each radioactive fragment in the termination reaction is reduced proportionately.

If sequences 300 to 1000 nucleotides from the primer are of primary interest, then either the concentration of the four dNTPs in the labeling reaction can be raised (by using the long labeling mixes and increasing the amount of [α - ^{35}S]dATP) or the dNTP:ddNTP ratio in the termination mixes can be increased (e.g., by a factor of 2 to 3).

Using the short and long labeling mixes, respectively, 3 pmol and 15 pmol of each nucleotide are added to the labeling reaction. The amount of [α - ^{35}S]dATP included should not be lower than these values so that it will not become the limiting nucleotide. Amounts greater than these are not incorporated in the labeling reaction since the other three dNTPs become limiting. However, we have found empirically that adding an excess of [α - ^{35}S]dATP (such as 40 pmol, or 2 μ l of 500 Ci/mmol at 10 mCi/ml) to the labeling reactions containing the short mixes results in darker bands on the sequencing gel. This is probably because the [α - ^{35}S]dATP unused in the labeling reaction contributes a significant proportion of the dATP in the termination reactions. We recommend using only the appropriate amount of [α - ^{35}S]dATP in the labeling reactions to avoid generating more radioactive waste than neces-

sary and because the labeled nucleotide is a significant fraction of the cost of sequencing reagents.

Factors affecting extension length in the Sanger and thermal cycle sequencing procedures

Because extension and termination occur in the same step in the Sanger and thermal cycle sequencing procedures, the average length of dideoxy-terminated fragments can only be adjusted by changing the ddNTP/dNTP ratio in the sequencing mixes. Changing the extension length in the Sanger reaction is most common when sequence information close to the primer is of interest. In this case the ddNTP:dNTP ratios can be increased ~5-fold, usually by increasing ddNTP concentrations. Conversely, decreasing the ddNTP concentration will effectively lengthen the sequence that can be read, while decreasing the frequency of stops at the bottom part of the sequence. If the template is particularly rich in one nucleotide, depletion of the complementary nucleotide in that reaction can occur (because the dNTP concentration is lower in that reaction mix) and the sequencing ladder for that reaction may "fade out" at the top. In these cases, the ddNTP/dNTP ratio can be individually adjusted (e.g., by raising the dNTP concentration in that mix).

Troubleshooting

Refer to Table 7.4.3 for a guide to troubleshooting general problems that are encountered with dideoxy sequencing, and to tables 7.4.4, 7.4.5, and 7.4.6 for problems specific to the labeling/termination, Sanger, and thermal cycle sequencing procedures, respectively.

DNA preparations of poor quality are the most common problem encountered with sequencing reactions. Template impurities reduce the effectiveness of DNA polymerase in the sequencing reactions, resulting in higher levels of artifactual bands caused by premature termination. It is important to have highly purified control DNA for testing or troubleshooting reactions.

Denatured double-stranded DNA templates can give particularly troublesome backgrounds because these preparations are often contaminated with residual salts, proteins, and RNA. Moreover, nicks in the DNA can act as priming sites during the sequencing reaction. Using a 5'-end-labeled primer rather than uniform labeling with [α - ^{35}S]dATP often overcomes the background problems associated with nicked templates because only elongated primers are

detected (see one-step alternative protocol). Empirically, we have found that the greater the percentage of supercoiled molecules in a plasmid preparation, the lower the background on the sequencing gel. Thus, for example, we recommend using an *E. coli* strain which is EndoI⁻ to propagate the plasmid. Somewhat paradoxically, there are several reports in the literature that linearization of circular double-stranded DNA templates reduced artifactual bands (Gravel et al., 1985; Mierendorf and Pfeffer, 1987; Wallace et al., 1981).

Interpretation of sequencing gels

Refer to Figures 7.4.1 and 7.4.3 for examples of how to "read" dideoxy sequencing gels. Autoradiograms are read from bottom to top, i.e., in the direction of synthesis (5'→3'). The spacing between oligodeoxynucleotides that differ in length by only one nucleotide gradually becomes narrower towards the top of the gel. To read more sequence data at the top of the gel, a shorter exposure time is often best as this results in finer bands and a cleaner background. Use of a higher-contrast film such as Kodak OM-1, rather than the Kodak XAR-5 recommended in UNIT 7.6 can also help; however, the exposure time is about three times longer for OM-1 than for XAR-5 for a comparable exposure.

Dark "shadow" or "ghost" bands may become more of a problem with long exposure times. By paying careful attention to spacing between oligodeoxynucleotides and considering enzymatic artifacts described in the previous section, it is usually possible to determine what is a shadow band and what is not.

Compressions

The largest single problem encountered in both the dideoxy and chemical sequencing methods is that of compressions in the banding pattern. Compressions are electrophoretic artifacts that occur when the bases of the single-stranded product of the sequencing reaction interact to form secondary structures, such as hairpins, that are stable even in the 7 M urea that is included in the sequencing gel. Secondary structures are most likely to form in regions of DNA that contain extended palindromic sequences or that are rich in G and C residues.

In practice, the migration of an oligonucleotide in a sequencing gel is affected by secondary structure only when the region of DNA capable of forming that structure is found at the 3' end of the oligonucleotide. This explains the observation that the migration of only a few

adjacent fragments in the sequencing ladder is altered by a sequence that can form a secondary structure. The result is that any given compression occurs only in a limited region of the gel. Anomalous spacing of bands, extra bands, or missing bands are all important clues that a compression has occurred. A typical compression pattern consists of comigration of bands in different lanes and anomalously wide spacing of bands above this region (Fig. 7.4.1). However, it is sometimes nearly impossible to identify a compression from the sequence of one strand alone.

A compression does not typically occur at the same site on both strands. When a compression does occur, the region that will be compressed on the opposite strand (if the complementary secondary structure is stable) will be on the other side of the secondary structure. This is because the secondary structure cannot form until the nucleotides involved in forming the secondary structure have been added to the growing oligonucleotide chain. Thus, the best criterion for identifying compressions is any discrepancy in the sequence between the two strands. Analysis of the discrepancies and the potential secondary structure of the neighboring sequence often reveals the site of compression and the true sequence of the region.

A variety of methods have been used to eliminate compressions. A very simple solution that may resolve some compressions is to run the gels at the highest possible temperature. This approach is limited, however, by the tendency of the glass gel plate to crack at high temperatures and by reduction in the resolution of the gel at high temperatures, leading to "fuzzy" bands.

Inclusion of formamide in the sequencing gel promotes denaturation of secondary structures and often solves many compression problems (Fig. 7.4.3; Martin, 1987; Brown, 1984; U.S. Biochemical, 1990). If this approach is tried, include 25% to 40% formamide in the acrylamide gel solutions (see UNIT 7.6).

A third method for eliminating compressions, not widely used, is to chemically modify C residues so that they can no longer engage in the formation of G-C base pairs. This can be accomplished by treating the synthesized oligonucleotides with either bisulfite or a mixture of bisulfite and methoxyamine (Ambartsumyan and Mazo, 1980; Hayatsu, 1976). These three procedures for eliminating compressions are equally applicable to enzymatic or chemical sequencing methods (UNIT 7.5).

For enzymatic sequencing, the use of nucle-

otide analogs that have a lower tendency to form secondary structures is frequently an effective method for eliminating compressions (Fig. 7.4.1). Deoxyinosine-5'-triphosphate (dITP) has been used traditionally for this purpose (Mills and Kramer, 1979). Inosine is an analog of guanosine that bonds considerably more weakly to cytidine. The concentrations in the nucleotide mixes must be adjusted to compensate for the fact that DNA polymerases use

dITP less efficiently than dGTP. dITP is substituted for dGTP in the nucleotide mixes but ddGTP is still used (not ddITP; see alternate protocols; Bankier and Barrell, 1983).

When Sequenase is used in sequencing reactions containing dITP, some fragments are susceptible to degradation by a reaction called pyrophosphorolysis (see below). The presence of inorganic pyrophosphatase prevents this degradation (Tabor and Richard-

Table 7.4.3 General Troubleshooting Guide for Dideoxy Sequencing

Problem	Possible cause	Solution
Blank autoradiogram	Inactive polymerase Old label Old reagents Incorrect or defective primer Incorrect or defective template	Test using control primer, template, and reagents. Replace defective reagent.
	Incorrect exposure procedure (plastic wrap left on, gel facing backwards, etc.)	Correct error
Entire autoradiogram too light/poor incorporation of label	Old label	Obtain new label
	Low enzyme activity	Obtain fresh preparation of enzyme. Dilute immediately before use and keep on ice.
	Primer did not anneal well to template	See critical parameters for calculating maximum annealing temperature for primers. If template is double-stranded, remember to denature it in alkali before annealing (UNIT 7.3).
	Not enough primer	Test using control DNA and primer. Check concentration of primer. Use 0.5 pmol/single-stranded template, 1 pmol/double-stranded template.
	Not enough template	Test using control DNA and primer. Check template concentration on gel or by other method. Use ~0.5 pmol template DNA per set of reactions.
	Incorrect exposure or development of autoradiogram	Reexpose gel. Change developing reagents, if necessary.
	Failure to remove plastic wrap from gel if using ³⁵ S	Remove plastic wrap
High background in all lanes	Impure template DNA	See if problem persists with control DNA. If not, make new template DNA. For single-stranded templates, try optional annotations in UNIT 7.3. For double-stranded templates, treat with RNase A (UNIT 3.13), phenol extract and ethanol precipitate (UNIT 7.3). If necessary, CsCl-purify DNA.

continued

Table 7.4.3 General Troubleshooting Guide for Dideoxy Sequencing, continued

Problem	Possible cause	Solution
Bands throughout the lanes are diffuse or fuzzy	Impure template DNA	See if problem persists with control DNA. If not, make new template DNA. For single-stranded templates, try optional annotations in protocol, <i>UNIT 7.3</i> . For double-stranded templates, treat with RNase A, phenol extract and ethanol precipitate as described in <i>UNIT 7.3</i> . If necessary, CsCl-purify the DNA.
	Poor quality of acrylamide gel	Prepare fresh acrylamide, bisacrylamide, and buffers using only high-quality reagents. Store stock solutions at 4°C in dark (<i>UNIT 7.6</i>).
	Gel used too soon after pouring	Let gel set up longer
	Buffer concentration in gel differs from concentration in reservoirs	Prepare gel and reservoir solutions from same 10× TBE stock
	Excessive boiling of samples	If samples are to be loaded on multiple gels, remove 3-μl aliquot of each reaction for heating and loading on gel
	Samples not denatured before running on gel	Heat samples in 95°C water bath 2 to 3 min
	Gel electrophoresis at too high a temperature	Monitor gel temperature with thermometer, keep temperature below 65°C. If necessary, run gel at lower wattage to keep temperature lower.
Areas on gel where bands are fuzzy	Gel or plastic wrap on top of gel dried with wrinkle	See <i>UNIT 7.6</i> , processing sequencing gels, for suggestions to avoid wrinkles
	Film was not clamped tightly to the gel	Insert filter paper behind gel to take up any extra space in X-ray cassette. Use more clamps, if necessary.
Distortion of all bands in 450-550-nucleotide region of the gel	>0.5% glycerol in samples (Tabor and Richardson, 1987b)	Dilute DNA polymerase in diluent without glycerol
Bands in all four lanes over entire gel	Low enzyme activity	Use fresh enzyme preparation; dilute immediately before use in appropriate diluent or 1× sequencing buffer
	Incorrect buffer or nucleotide mixes	Prepare new mixes
	Contaminated reagents	Prepare fresh reagents
Anomalous spacing of bands, missing bands, or bands at the same position in two or three lanes only at specific regions	Compression (due to secondary structure of newly synthesized DNA strands under conditions of gel electrophoresis)	See commentary for a discussion of various methods for eliminating compressions
Bands at the same position in more than one lane throughout the gel	DNA preparation contains two different DNAs that are producing overlapping sequences	Prepare new DNA starting from a single plaque or colony
	Primer has annealed to secondary sites on template	See critical parameters for discussion of primer/template ratio and annealing temperature

Table 7.4.4 Guide to Troubleshooting in the Labeling/Termination Protocol

Problem	Possible cause	Solution
Bands at the same position in all four lanes, especially near the primer	Impure template DNA ^a	See if problem persists with control DNA. If not, make new template DNA.
	Impure or old reagents; not enough dNTPs ^a	Prepare fresh reagents
	DNA polymerase activity low ^a	(1) Use fresh enzyme preparation. (2) Add more enzyme, e.g., four times as much per reaction. (3) Dilute enzyme immediately before use. (4) Reduce labeling reaction to 2 min to minimize the chance of T7 DNA polymerase dissociating from template.
Bands below a certain site on the gel are very dark, and bands above that are very faint	Secondary structure of the template impeding the extensions in the labeling step	Alter the conditions of the labeling reaction so that the extensions in the labeling reaction do not reach the secondary structure: (1) lower the concentration of the 3 dNTPs in the labeling mix, e.g., dilute 4-fold; (2) reduce the time of the labeling reaction to 1 or 2 min; (3) raise the DNA concentration 2- to 3-fold
		Use higher reaction temperature with alternate polymerase (and maybe alternate protocol) such as thermophilic polymerase. Refer to commentary for discussion of alternate polymerases.
		Add 0.5 µg <i>E. coli</i> single-stranded DNA binding protein to the labeling reaction to help the polymerase synthesize through regions of secondary structure. The binding protein must be removed by treating with 0.1 µg of proteinase K at 65°C for 20 min after adding formamide/dye stop solution.
Intensity of bands at the bottom of gel is too low	Proportion of extensions that have terminated near the bottom of the gel is too low	Reduce the concentration of the labeling mix in the labeling reaction, e.g., dilute an additional 3- to 5-fold
		Reduce labeling time to 1 to 2 min
		Raise DNA concentration 2- to 3-fold
Intensity of bands at the top of the gel is too low	The polymerase is incorporating a ddNMP before the DNA fragments have reached the appropriate length	Decrease ratio of dNTP to ddNTP in the termination reaction, e.g., raise the ddNTP concentration 2-fold
		Use a primer that lies further upstream of the site to be sequenced
		Raise the concentration of the labeling mix in the labeling reaction (e.g. by using "long" mixes)
Range of bands that can be sequenced is narrow (radioactivity is restricted to bands spanning 200 nucleotides)	DNA concentration in the sequencing reaction is low	Increase the dNTP/ddNTP ratio in the termination, e.g., reduce the ddNTP concentration in each termination mix 2-fold
		Increase the DNA concentration in the sequencing reaction to 2 µg

^aThis may occur because of enzyme pausing during the labeling reaction and not reinitiating synthesis efficiently.

Table 7.4.5 Guide to Troubleshooting in the Sanger Procedure

Problem	Possible cause	Solution
High background in "A" track, especially when [³⁵ S]dATP is older than one half-life	Old label	Replace with fresh label, or reduce label in reactions
Intensity of bands is too low at top of gel	Ratio of ddNTP to dNTP is too high	If problem is specific to one mix make up new nucleotide mix; otherwise decrease ddNTP/dNTP ratio by lowering ddNTP in each mix
Intensity of bands is too low at bottom of gel	Ratio of ddNTP to dNTP is too low	If problem is specific to one mix, make up new mix; otherwise increase ddNTP/dNTP ratio by increasing ddNTP in each mix

son, 1990). Pyrophosphatase should always be present when dITP is used in the labeling/termination procedure with modified T7 DNA polymerase. A mixture of modified T7 DNA polymerase and yeast inorganic pyrophosphatase can be mixed together and stored in 50% glycerol at -20°C (see reagents and solutions). These enzymes are stable together for several years. Sequencing reactions using dITP should be performed in parallel with reactions containing dGTP because dITP-containing reactions tend to have banding artifacts caused by premature termination of the polymerase.

An alternative to dITP that is effective in reducing band compressions in some cases is 7-deaza-dGTP (7-deaza-2'-deoxyguanosine-5'-triphosphate; Seela et al., 1982; Mizusawa et al., 1986; Barr et al., 1986). 7-deaza-dGTP, which has a methine moiety at the N-7 position of the guanine nucleus, has been suggested to reduce compressions by disrupting the formation of alternative Hoogsteen base pairs (Mizusawa et al., 1986; Sarecchi et al., 1970). However, it is possible that 7-deaza-dGTP also interferes with standard Watson-Crick base pairing by base pairing more weakly with cytidine. 7-deaza-dGTP is used by Sequenase, Klenow fragment, *Taq* polymerase, Vent_R polymerase, and reverse transcriptase more efficiently than dITP; it can be substituted at a 1:1 molar ratio for dGTP in the sequencing reactions (see alternate protocols). However, it may not be as effective as dITP at removing many compressions (Fig. 7.4.1; Tabor and Richardson, 1987b). 7-deaza-dATP in combination with 7-deaza-dGTP may also be useful in resolving compressions (Pharmacia, 1991).

Premature termination

Stretches of single-stranded DNA that are rich in G + C or A + T or that are extensively palindromic can be particularly difficult to sequence accurately, not only because of stable secondary structure formation in the products on the gel (compressions) but also because of stable secondary structure in the template during the sequencing reaction. When bands in all four lanes are seen at or shortly before such a sequence and the sequencing ladder is very faint after that point, it is likely that the template secondary structure in the sequencing reaction has prevented the DNA polymerase from advancing past the "foldback" position (Fig. 7.4.3). In such cases, using more polymerase can be helpful. Another strategy is to perform sequencing reactions at a higher temperature to destabilize the template secondary structure. The thermophilic DNA polymerases, such as *Taq* DNA polymerase, are the enzymes of choice in this situation (see above). An alternative strategy is to include *E. coli* single-stranded DNA binding protein in the sequencing reactions to destabilize the secondary structures (Table 7.4.4). For templates with severe secondary structure problems, chemical sequencing (UNIT 7.5) can be employed.

Premature termination can also result in "ghost" or "shadow" bands or bands in all four lanes. This can arise from a variety of causes, such as impure template DNA, use of dITP (Fig. 7.4.3), or performing reactions at a temperature at which the polymerase rapidly loses activity. One group has reported improvement of "shadow" bands when using Sequenase in the labeling/termination protocol by addition

Table 7.4.6 Guide to Troubleshooting in the Thermal Cycle Sequencing Protocols

Problem	Possible cause	Solution
Bands are seen at same position in all four lanes, especially near primer.	Impure template DNA	See if problem exists with control DNA. If not, repurify DNA.
	Incorrect primer annealing temperature	Reduce primer annealing temperature; use longer (more stable) primer; increase annealing and extension steps to 1 min each
	Impure or old reagents; ddNTP amount too high or dNTP amount too low	Prepare fresh reagents and readjust dNTP/ddNTP ratios
Bands below a certain site on gel are very dark, and bands above that are very faint.	Secondary structure of template impeding extensions	Use higher reaction temperatures
A dark band is present across all 4 lanes of gel; bands below and above it are equally dark.	Secondary structure of reaction products causing anomalous electrophoresis position of products in gel	Use higher gel temperatures or formamide gel; use base analogs or TdT in reaction; for PCR products, check purification procedure
Intensity of bands at bottom of gel is too low.	Proportion of reaction products terminating near bottom of gel too low	Increase ddNTP:dNTP ratio in sequencing mixes
Intensity of bands at top of gel is too low.	Proportion of reaction products terminating near top of gel too low	Decrease ddNTP:dNTP ratio in sequencing mixes
One lane of reaction failed or is weak or smeary.	Possible cycler fault in one reaction slot	Check cycler performance
	Pipetting error	Review chemistry procedure
All lanes are smeary or show high backgrounds.	Template impurity	Review template preparation; run control DNA template
	Primer impurity	Check primer purity
	Cycler problem	Check cycler function
	Reagent problem	Review chemistry procedure; make fresh reagents
Entire sequence is light.	Primer/template ratio too high	Review chemistry procedure
	Incorrect priming temperature	Calculate T_m as guideline for reaction
	Insufficient template or primer in reaction	Double DNA and primer quantities in reaction
Oil covering reaction makes it difficult to load gel.	Cycler error; incorrect or inadequate cycling conditions	Check cycler performance; make steps 1 min instead of 30 sec; increase cycles to 30
	Excess oil present	Remove oil or use hot top-apparatus (see commentary); microcentrifuge reaction before loading gel

of terminal deoxynucleotidyl transferase (TdT; UNIT 3.6) to the sequencing reactions (Fawcett and Bartlett, 1990). TdT, in the presence of dNTPs, extends chains which have not been terminated by incorporation of a ddNMP in a template-independent process to higher-molecular-weight DNA.

Pyrophosphorolysis

During DNA synthesis, the incorporation of dNMPs results in the accumulation of inorganic pyrophosphate. At a high enough level, the inorganic pyrophosphate can drive the reverse reaction of polymerization, called pyrophosphorolysis. Pyrophosphorolysis can lead to the disappearance of specific fragments on a DNA sequencing gel with some DNA polymerases (e.g., Sequenase; Tabor and Richardson, 1990). The removal of a dideoxynucleotide by pyrophosphorolysis leaves a chain with a normal 3'-hydroxyl group that is then rapidly extended by the DNA polymerase. This results in bands that are missing from the sequencing ladder or whose intensity is weak. Pyrophosphorolysis is most noticeable when using Sequenase, and particularly in reactions in which dITP has been substituted for dGTP. A simple solution to this problem is the inclusion of inorganic pyrophosphatase in the sequencing reaction. It is strongly recommended that inorganic pyrophosphatase be present in all sequencing reactions using Sequenase; under these conditions, all bands are stable to incubation of the sequencing reactions for at least 1 hr (Tabor and Richardson, 1990). Alternatively, when using Sequenase in the absence of inorganic pyrophosphatase, incubation of the termination reactions should be kept to a minimum (5 min), particularly when using dITP. Pyrophosphorolysis has also been observed with AMV reverse transcriptase-catalyzed sequencing reactions (Ruan et al., 1990).

Time Considerations

The dideoxy sequencing protocol can be carried out in a single long day. In general, we recommend that if a large region of DNA is to be sequenced, the sequencing should be carried out in a concerted effort. DNA sequencing is easier, more efficient, and more successful when a daily rhythm has been established.

A day's work usually starts with pouring the sequencing gels. Then, template-primer annealing reactions are set up. During the annealing reactions, there is usually time to develop the autoradiograms of the sequencing gels from the previous day. Next, the primer extension

reactions are started. When these are complete, the reactions are loaded onto the gels. After lunch, the gels are stopped, dried down, and put on film overnight. The day ends by cleaning the gel plates and setting them up for the next day. The whole procedure takes 7 to 8 hr for 10 templates, 9 to 10 hr for 20 templates.

It is possible to split the protocol into 2 days. On day 1, the sequencing reactions are carried out, stop/loading dye is added, and the mixtures are stored overnight at -20°C . The sequencing gels can also be poured and stored overnight (see UNIT 7.6). The next day, the samples are defrosted, heated to 95°C for 2 min, and loaded on the sequencing gels. Samples containing uniform-labeled ^{35}S can be stored up to one week. Samples containing uniform-labeled ^{32}P should be run on the gel the same day; in this case only gels can be prepared on day 1.

The same schedule may be followed for thermal cycle sequencing. Alternatively, the gel may be poured in the afternoon and the cycling reactions set up to be run overnight. The following morning, the gel is run and processed and the procedure from the previous day is repeated as the gel is analyzed.

Literature Cited

- Ambartsumyan, N.S. and Mazo, A.M. 1980. Elimination of the secondary structure effect in gel sequencing of nucleic acids. *FEBS Lett.* 114:265-268.
- Bankier, A.T. and Barrell, B.G. 1983. Shotgun DNA sequencing. In *Techniques in Life Sciences, B5: Nucleic Acid Biochemistry* (R.A. Flavell, ed.) pp. 1-34. Elsevier/North Holland, Limerick, Ireland.
- Barr, P., Thayer, R., Najarian, R., Seela, F., Laybourn, P., and Tolan, D. 1986. 7-deaza-2'-guanosine triphosphate: Enhanced resolution in M13 dideoxy sequencing. *BioTechniques* 4:428-432.
- Bartlett, J., Gaillard, P., and Joklik, W. 1986. Sequencing of supercoiled plasmid DNA. *BioTechniques* 4:208-210.
- Brow, M.A.D. 1990. Sequencing with *Taq* DNA polymerase. In *PCR Protocols: A Guide to Methods and Applications* (M. Innis, D.H. Gelfand, J.J. Snisky and T.J. White, eds.) pp. 189-196. Academic Press, San Diego.
- Brown, N. 1984. DNA sequencing. *Methods Microbiol.* 17:259-313.
- Derbyshire, V., Freemont, P.S., Sanderson, M.R., Beese, L., Friedman, J.M., Joyce, C.M., and Steitz, T.A. 1988. Genetic and crystallographic studies of the 3', 5'-exonucleolytic site of DNA polymerase I. *Science* 240:199-201.

- Fawcett, T.W. and Bartlett, S.G. 1990. An effective method for eliminating "artefact banding" when sequencing double-stranded DNA templates. *BioTechniques* 9:46-48.
- Gravel, R.A., Quan, F., and Korneluk, R.G. 1985. Rapid and reliable dideoxy sequencing of double-stranded DNA. *Gene* 40:317-323.
- Gyllenstein, U.B. 1989. PCR and DNA sequencing. *BioTechniques* 7:700-708.
- Hayatsu, H., 1976. Bisulfite modification of nucleic acids and their constituents. *Prog. Nucleic Acid Res. Mol. Biol.* 16:75-124.
- Innis, M., Myambo, K., Gelfand, D., and Brown, M. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. U.S.A.* 85:9436-9440.
- Jack, W., Kucera, R., and Kong, W. 1992. Biochemical characterization of Ventr and Deep Ventr DNA polymerase. *NEB Transcript (New England Biolabs)* 4:4-5.
- Kong, H., Kucera, R., and Jack, W. 1992. Characterization of a DNA polymerase from the hyperthermophile Archae *Thermococcus litoralis*. Submitted for publication.
- Krawetz, S. 1987. DNA sequencing with AMV (avian myeloblastosis virus) reverse transcriptase and a modified T7 polymerase (Sequenase). *BioTechniques* 5:620-627.
- Mardis, E.R. and Roe, B.A. 1989. Automated methods for single-stranded DNA isolation and dideoxynucleotide DNA sequencing reactions on a robotic workstation. *BioTechniques* 7:840-850.
- Martin, R. 1987. Overcoming DNA sequencing artifacts: Stops and compressions. *Focus (BRL)* 9(1):8-10.
- McClary, J., Ye, S., Hong, G.F., and Whitney, F. 1991. Sequencing with the large fragment of DNA polymerase I from *Bacillus stearothermophilus*. *J. DNA Map. Seq.* 1:173-180.
- Mead, D.A., McClary, J.A., Luckey, J.A., Kostichka, A.J., Whitney, F.R., and Smith, L.M. 1991. *Bst* polymerase permits rapid sequence analysis from nanogram amounts of template. *BioTechniques* 11:76-87.
- Mierendorf, R.C. and Pfeffer, D. 1987. Direct DNA sequencing of denatured plasmid DNA. *Methods Enzymol.* 152:556-562.
- Mills, D.R. and Kramer, F.R. 1979. Structure independent nucleotide sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 76:2232-2235.
- Mizusawa, S., Nishimura, S., and Seela, F. 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. *Nucl. Acids Res.* 14:1319-1324.
- Perler, F., Comb, M., Jack, W., Moran, L., Qiang, B.-Q., Kucera, R., Benner, J., Slatko, B., Nwankwo, D., Hempstead, K., Carlow, P., and Jannasch, H. 1992. Intervening sequences in an Archae DNA polymerase gene. *Proc. Natl. Acad. Sci. U.S.A.* 89:5577-5581.
- Peterson, M. 1988. DNA sequencing using *Taq* polymerase. *Nucl. Acids Res.* 16:10915.
- Pharmacia. 1991. New! 7-deaza-2'-deoxyadenosine triphosphate (c7 dATP) *Analects* 19(4):3-4.
- Ross, J. and Leavitt, S. 1991. Improved sample recovery in thermo cycle sequencing protocols. *BioTechniques* 11:618-619.
- Ruan, C.C., Samols, S.B., and Fuller, C.W. 1990. Role of pyrophosphorolysis in DNA sequencing. *Comments (U.S. Biochemical)* 17(1):1-27.
- Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467.
- Sanger, F. and Coulson, A.R. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* 87:107-110.
- Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161-178.
- Sarecchi, M.-T., Courtois, Y., and Guschlbauer, W. 1970. Protonated polynucleotide structures: Specific complex formation between polycytidylic acid and guanosine or guanylic acids. *Eur. J. Biochem.* 14:411-421.
- Sears, L.E., Moran, L.S., Kissinger, C., Creasey, T., Perry-O'Keefe, H., Roskey, M., Sutherland, E., and Slatko, B.S. 1992. CircumVent thermal cycle sequencing and alternative manual and automated DNA sequencing protocols using the highly thermostable Ventr (exo⁻) DNA polymerase. *BioTechniques* 13:626-633.
- Seela, F., Tran-Thi, G.-H., and Franzen, D. 1982. Poly (7-deazaquanylic acid), the homopolynucleotide of the parent nucleoside of queuosine. *Biochemistry* 21:4338-4343.
- Seela, F., Berg, H., and Rosemeyer, H. 1989. Bending of oligonucleotides containing an isosteric nucleobase: 7-deaza-2'-deoxyadenosine replacing dA within d(A)₆ tracts. *Biochemistry* 28:6193-6198.
- Slatko, B.E. 1991a. Protocols for manual dideoxy DNA sequencing. In *Methods in Nucleic Acids Research* (J. Karam, L. Chao, and G. Warr, eds.) pp. 83-129. CRC Press, Boca Raton, Fla.
- Slatko, B.E. 1991b. Sources of reagents and supplies for dideoxy DNA sequencing and other applications. In *Methods in Nucleic Acids Research* (J. Karam, L. Chao, and G. Warr, eds.) pp. 379-392. CRC Press, Boca Raton, Fla.
- Stambaugh, K. and Blakesley, R. 1988. Extended DNA sequencing with Klenow fragment: The Kilobase Sequencing system. *Focus (BRL)* 10(2):29-51.

- Stoflet, E., Koeber, L., Sarkar, and Sommer, S. 1988. Genomic amplification with transcript sequencing. *Science* 239:491-494.
- Tabor, S. and Richardson, C.C. 1987a. Selective oxidation of the exonuclease domain of bacteriophage T7 DNA polymerase. *J. Biol. Chem.* 262:15330-15333.
- Tabor, S. and Richardson, C.C. 1987b. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 84:4767-4771.
- Tabor, S. and Richardson, C.C. 1989a. Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by in vitro mutagenesis. *J. Biol. Chem.* 264:6447-6458.
- Tabor, S. and Richardson, C.C. 1989b. Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *E. coli* DNA polymerase I. *Proc. Natl. Acad. Sci. U.S.A.* 86:4076-4080.
- Tabor, S. and Richardson, C.C. 1990. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase: Effect of pyrophorolysis and metal ions. *J. Biol. Chem.* 265:8322-8328.
- U.S. Biochemical. 1990. Formamide gels (40%) for sequencing DNA. *Comments* 17(1):31.
- Van Zeeland, A., Vrieling, H., Simons, J., and Lohman, P. 1990. Sequence determination of point mutations at the HPRT locus in mammalian cells following in vitro amplification of HPRT cDNA prepared from total cellular RNA. In *Current Communications in Molecular Biology: Polymerase Chain Reaction* (H. Ehrlich, P. Gibbs, and H. Kazazian, eds.) pp. 119-124. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Wallace, R.B., Johnson, M.J., Suggs, S.V., Miyoshi, K., Bhatt, R., and Itakura, K. 1981. A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. *Gene* 16:21-26.
- Whitehouse, E. and Spears, 1991. A simple method for removing oil from cycle sequencing reactions. *BioTechniques* 11:616-618.
- Williams, S.A., Slatko, B.E., Moran, L.S., and DeSimone, S.M. 1986. Sequencing in the fast lane: A rapid protocol for [α - 32 S]dATPideoxy sequencing. *BioTechniques* 4:138-147.
- Ye, S. and Hong, G. 1987. Heat stable DNA polymerase I large fragment resolves hairpin structure in DNA sequencing. *Sci. Sin.* 30:503-506.
- Zagursky, R.J., Baumeister, K., Lomax, N., and Berman, M.L. 1985. Rapid and easy sequencing of large double-stranded DNA and supercoiled plasmid DNA. *Gene Anal. Tech.* 2:89-94.

Key References

- Tabor and Richardson, 1987b, 1990. See above.
Describes the procedure for DNA sequence analysis with Sequenase.
- Sanger et al., 1977. See above.
Describes the Sanger protocol using Klenow fragment.
- Sears et al., 1992. See above.
Describes the thermal cycle reaction.

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Front cover (paperback): The gene encoding green fluorescent protein was cloned from *Aequorea victoria*, a jellyfish found in abundance in Puget Sound, Washington State. This picture of a 50-mm medusa was taken on color film by flash photography and shows light reflected from various morphological features of the animal. The small bright roundish blobs in the photograph are symbiotic amphipods living on or in the medusa. The bright ragged area in the center is the jellyfish's mouth.

Bioluminescence from *Aequorea* is emitted only from the margins of the medusae and cannot be seen in this image. Bioluminescence of *Aequorea*, as in most species of jellyfish, does not look like a soft overall glow, but occurs only at the rim of the bell and, given the right viewing conditions, would appear as a string of nearly microscopic fusiform green lights. The primary luminescence produced by *Aequorea* is actually bluish in color and is emitted by the protein aequorin. In a living jellyfish, light is emitted via the coupled green fluorescent protein, which causes the luminescence to appear green to the observer.

The figure and legend were kindly provided by Claudia Mills of the University of Washington, Friday Harbor. For further information, please see Mills, C.E. 1999–2000. Bioluminescence of *Aequorea*, a hydromedusa. Electronic Internet document available at <http://faculty.washington.edu/cemills/Aequorea.html>. Published by the author, web page established June 1999, last updated 23 August 2000.

Back cover (paperback): A portion of a human cDNA array hybridized with a red fluor-tagged experimental sample and a green fluor-tagged reference sample. Please see Appendix 10 for details. (Image provided by Vivek Mittal and Michael Wigler, Cold Spring Harbor Laboratory.)

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TABLE 12-13 Troubleshooting: Problems That Commonly Arise during Dideoxy-DNA Sequencing

SYMPTOM	POSSIBLE CAUSE	QUESTIONS TO ASK	POSSIBLE REMEDIES
1. Faint bands or no bands at all are visible on the autoradiograph.	<p>An essential component (enzyme, dNTPs, primer radioactive precursor) was omitted from reaction.</p> <p>Poor annealing of primer.</p> <p>Saran Wrap was not removed from gel labeled with ^{32}P or ^{35}S.</p> <p>Film processor is broken.</p>	<p>Was the correct labeled dNTP used? Was the specific activity of the labeled dNTP correct?</p> <p>Was the dNTP used within two half-lives?</p> <p>Was a control template included in the experiment?</p> <p>Was the correct primer used? Does the primer have the correct sequence and orientation?</p>	<p>If the lanes containing the control template were faint or blank and if the correct radiolabel was used, the most likely cause of this type of catastrophic failure is the labeling mixture, the DNA polymerase, or the chain extension/termination mixture. Repeat the experiment using new reaction mixtures, a new batch of enzyme, and a positive control template.</p> <p>Check that the film processor is fully functional, that the correct primer is used, and that the gel is correctly processed.</p>
2. Bands are faint at top of gel.	<p>Template DNA is impure.</p> <p>Too much template was used.</p> <p>Ratio of ddNTP to dNTP in the extension/termination mixture was too high.</p>	<p>Was a control template included in the experiment?</p> <p>Was the chain termination/extension mixture appropriate for the DNA polymerase used to catalyze the sequencing reaction?</p>	<p>The key to diagnosis is the control template. If lanes containing the control template show the same symptoms, the most likely cause of the problem is the ratio of ddNTP to dNTP in the extension/termination mixture. Check that the mixture used was appropriate for the sequencing enzyme. Repeat the experiment with a new batch of extension/termination mixture.</p> <p>If lanes containing the control template do not show the symptom, the templates in the remaining lanes must be contaminated with inhibitors. The remedy is to purify the templates further (proteinase K digestion, phenol extraction, and ethanol precipitation) and to redissolve them in H_2O rather than TE.</p>
3. Bands become fainter toward bottom of the gel.	Ratio of ddNTP to dNTP was too low.	<p>Were radiolabeled dNTPs used as precursors in cycle-sequencing reactions or in isothermal reactions catalyzed by <i>Taq</i> DNA polymerase or the Klenow fragment?</p> <p>Was the chain termination/extension mixture appropriate for the DNA polymerase used to catalyze the sequencing reaction?</p>	<p>Repeat the experiment using a new batch of chain termination/extension mixture.</p> <p>The best remedy is to change to an end-labeling protocol. Otherwise, repeat the reactions using twice as much labeled dNTP and be prepared to expose the dried gel to film for a long time.</p>
4. One set of sequencing reactions yields four blank tracks.	Template DNA was omitted accidentally, or was degraded, or contains a powerful inhibitor of the sequencing reaction (e.g., EDTA), or does not contain an appropriate primer-binding site.	Were the concentration and integrity of the template verified on an agarose gel?	<p>Purify the template further (proteinase K digestion, phenol extraction, and ethanol precipitation) and redissolve it in H_2O rather than TE.</p> <p>Use another primer that binds to a different site in the template DNA.</p>

5. Three of the four sequencing reactions work well with all templates (including the control) but one lane is always faint or blank.

One of the four chain extension/termination mixtures is defective.

Was the frozen stock of extension/termination mixture maltreated, e.g., by thawing in a water bath? Or was it left at room temperature after thawing?
6. Amount of radiolabel incorporated during the sequencing reaction varies from template to template.

Templates are present at different concentrations.

Template DNAs are partially degraded, or they contain an inhibitor of the sequencing reaction (e.g., EDTA).
7. High background of radiolabeled bands in all four lanes makes the sequences difficult to read.

Template DNAs are impure.

Samples were heated too long before loading or, if labeled with ^{32}P , may have undergone radiolysis during storage.

Gel plates contain a residue of silanizing agent.

DNA polymerase has deteriorated or its concentration is limiting.

Samples evaporated during incubation.

Was a control template included in the experiment?

Is a smear of chromosomal DNA visible when the template DNAs are analyzed by agarose gel electrophoresis?

How long were the samples boiled/stored before loading the gel?
8. Shadow bands or double bands are present in one set of sequencing reactions.

More than one template is present.

Primer is binding to more than one site.
9. Shadow bands are present in all sequencing reactions.

Concentration of primer is too high.

DNA polymerase has deteriorated or its concentration is limiting.

Titrate the primer, using a new preparation of DNA polymerase. If this does not solve the problem, purify the primer by polyacrylamide gel electrophoresis.

If using cycle sequencing, reduce the number of cycles to ~30.

Repeat the reactions using DNA polymerase that lacks 5'-3' exonuclease activity.

Please also see Symptom 7, above.

(Continued on following pages.)

Make a fresh batch of chain extension/termination mixture.

Compare the results obtained from both batches in reactions containing a control template.

If the A-track is causing the problem with all templates (including the control), the stock of dATP may be degraded. If the problem is confined to the test templates, the DNAs may be rich in A residues.

Check the concentrations of the template DNAs on agarose gels; make sure that all sequencing reactions contain approximately equal amounts of template. If necessary, purify a new batch of template DNAs, dissolving them in H_2O rather than TE.

If the control template is exempt from the problem, the homemade DNA templates are most likely contaminated by fragments of chromosomal DNA, which cannot be easily removed by further purification of the template DNAs. The best solution may be to isolate and more carefully purify a new set of template DNAs from freshly picked plaques or colonies.

If the background is high in lanes containing the control DNA, the samples may have been heated too vigorously or stored too long and/or the DNA polymerase may be anemic. Repeat the sequencing reactions using a new preparation of DNA polymerase. Make sure the reactions are covered with mineral oil before incubation in a thermal cycler. Transfer the samples to microfuge tubes and denature them by heating to 95°C for 5 minutes; chill the samples to 0°C and load them onto the gel within 20 minutes.

Make sure the gel plates are clean.

Repurify the template DNA from a freshly picked plaque or colony. If the problem persists, use another primer that binds to a different site in the template DNA.

TABLE 12-13 (Continued)

SYMPTOM	POSSIBLE CAUSE	QUESTIONS TO ASK	POSSIBLE REMEDIES
	<p>Primer is heterogeneous in length and/or the annealing step was carried out at suboptimal temperature.</p> <p>Samples were boiled too long or, if labeled with ^{32}P, may have undergone radiolysis during storage.</p> <p>DNA polymerase carrying 5'-3' exonuclease activity was used to catalyze the sequencing.</p>		
10. Bands are present in all four tracks in particular regions of sequence or bands are missing from particular regions of sequence.	<p>Template contains homopolymeric tracts or regions of stable secondary structure.</p>	Was the template generated by PCR? If so, a primer-dimer or other PCR artifact may have been carried over to the sequencing reaction and has become labeled.	<p>Sequence the complementary strand.</p> <p>Use a different DNA polymerase.</p> <p>Carry out chain extension/termination reactions at higher temperature.</p> <p>Substitute 7-deaza-dGTP for dGTP in ddNTP mixtures and substitute MnCl_2 for MgCl_2 in sequencing buffer (please see the information panel on COMPRESSIONS IN DNA SEQUENCING GELS).</p> <p>Increase the temperature of electrophoresis to 60°C. Alternatively, use a polyacrylamide gel containing 40% formamide.</p>
11. Bands are diffuse through the length of the gel.	<p>Polyacrylamide gel and/or electrophoresis buffer is defective.</p> <p>Samples contain high concentrations of salt.</p> <p>Urea is present in the loading area of the gel.</p> <p>Temperature of the gel is too high during electrophoresis and/or during drying.</p> <p>Poor contact between gel and film.</p>	Does the control template show the same problem?	<p>If the bands in the control template are sharp, the test templates probably contain too much salt. Repurify the template DNA from a freshly picked plaque or colony.</p> <p>If the bands in the template DNA are diffuse, make up new gel mixtures/electrophoresis buffer and new ammonium sulfate solution. The sequencing gel should polymerize rapidly (within 15 minutes). Make sure that the gel mixture is at room temperature when the gel is cast; otherwise, urea may precipitate. Make sure that the loading area of the gel is thoroughly washed before the samples are loaded.</p> <p>Make sure that the temperature of the gel during electrophoresis is ~60°C and that the temperature control on the gel dryer is set correctly.</p>

Make sure that the gel is in close contact with the film during exposure of the autoradiograph. If using single-sided film, make sure that the emulsion side faces the dried gel.

Repeat the sequencing reactions using a fresh batch of radiolabel.

Pour a new gel and wash wells thoroughly to remove urea, unpolymerized acrylamide, and loose fragments of polyacrylamide.

Make up new gel mixtures using freshly purified components.

Make sure that the gel mixture is at room temperature when gel is cast; otherwise, urea may precipitate.

Transfer the samples to microfuge tubes and denature them by heating to 95°C for 5 minutes; chill the samples to 0°C and load them onto the gel within 20 minutes.

Use a glycerol-tolerant gel or reduce the amount of glycerol in the samples. Please see the information panel on **GLYCEROL IN DNA SEQUENCING REACTIONS**.

Pour a new sequencing gel, using clean gel plates.

Pour a new sequencing gel, taking care to clamp the gel properly and to insert the comb to the correct depth.

Ammonium persulfate solution used to catalyze the polymerization of acrylamide was not fresh.

Radiolabeled dNTP has deteriorated during storage.

Sample wells were not thoroughly washed before loading.

Precipitation of borate or urea from the gel mixtures.

Samples were not properly denatured.

Excess glycerol is present in samples loaded onto the gel.

Gel plates were not cleaned properly or gel was poured too quickly.

Top of the gel was not tightly clamped and/or the comb was not inserted deeply enough into the slot.

12. Diffuse bands extending across all lanes of the gel (including the control) ~75 bases from the 3' end of the primer.

13. Wavy bands.

14. Black dots on the autoradiograph.

15. Radioactivity remained in the sample well.

16. Bands are distorted at the top of the gel.

17. Bubbles in gel.

18. Samples from adjacent lanes become mixed after loading.

AUTOMATED DNA SEQUENCING

From its inception, the dideoxy chain-termination method of DNA sequencing (Sanger et al. 1977a), with enzymatic reactions carried out in aqueous solvents at moderate temperatures, was an obvious candidate for automation. By the early 1980s, the development of automated sequencing was well under way, using DNA molecules labeled with fluorescent dyes rather than the traditional radioisotopes (Smith et al. 1986). By the middle of the decade, automated fluorescent sequencers had been developed in which the products of dideoxy-sequencing reactions were separated by gel electrophoresis, dye molecules were excited by a laser beam, and the fluorescent signals were amplified and detected by photomultiplier tubes (or a CCD camera in later models) (for reviews, please see Chen et al. 1991; Hunkapiller et al. 1991; Chen 1994). Because fluorescent signals could be detected and processed continuously, gels could be run for longer times and more data could be collected. Computer software identified each nucleotide based on the distinctive color (emission wavelength) of each different dye, to identify (call) each base according to the shape of the fluorescent peak and the distance between successive peaks.

Evolution of the Technology

The events leading to current automated sequencing technology are summarized in Figure 12-10. The powerful impact of technical advances on sequencing efficiency is evident from the improvement in the number of samples (and average read-length per sample) that can be achieved in 1 week by a skilled, dedicated investigator. Judged from today's standards, the capacity of automated sequencers available up to 1990 was low, and data were often variable and poor in quality (Martin and Davies 1986; Chen et al. 1991; Hunkapiller et al. 1991). However, there was a great leap forward when PCR was harnessed to the dideoxy method in "cycle sequencing" (Carothers et al. 1989), a process that linearly amplifies signals and thereby greatly augments the sensitivity of sequencing. Additional improvements in dye chemistry and modifications of DNA polymerases have been a further source of enormous progress throughout the 1990s. As a consequence, the rate of sequencing had increased ~30-fold by 1998, so that a single investigator in a well-equipped laboratory could handle 500 samples a week with an average read-length of 650 bases per sample. By the year 2000, that rate had increased by another order of magnitude in high-throughput facilities equipped with capillary-based sequencers and robotic tools.

Initially, there were two major types of commercial instruments, both of which used gel electrophoretic separation of DNA fragments, one using single-dye four-lane separation (Ansorge et al. 1987), and the other using four-dye single-lane separation (Smith et al. 1986). Because of their higher throughput, greater consistency, and lower sensitivity to electrophoretic artifacts, the single-lane instruments have gradually become the approach of choice. The current slab gel model ABI 377 automated sequencer, which dominated the commercial market during the late 1990s, has a capacity of 96 samples per run, with two to four runs a day (depending on the read-lengths desired).

Capillary-based equipment delivers six to eight runs a day while eliminating the tedious gel preparation and sample loading steps. Capillary electrophoresis (Swerdlow and Gesteland 1990; Smith 1991; Yan et al. 1996; Dovichi 1999) also offers other advantages over traditional slab gel systems.

- Because each capillary is very small in diameter (50–100 μm), heat generated during electrophoresis can be rapidly dissipated. Very high voltages can therefore be applied to achieve separation of the products of sequencing reactions in a shorter period of time.
- Because each sample is loaded into a discrete capillary, there is no need for time-consuming tracking of lanes on gel images.

As of early 2000, three 96-channel, one 8-channel, and one 1-channel capillary-based sequencers were available (Boguslavsky 2000): MegaBACE (96-channel, Molecular Dynamics, California), ABI 3700 and ABI 310 (96-channel and 1-channel, respectively; PE Biosystems), SCE9610 (96-channel, Spectru-Medix, Pennsylvania), and CEQ 2000 (8-channel, Beckman Coulter, California). The ABI 3700 model is also equipped with an automated loader, permitting >6 runs a day unattended by an operator, or a capability of >4000 samples a week. Including the resultant savings in person power, this machine can increase sequencing efficiency by tenfold from the operations using previous models and has the potential to reduce the cost per finished base by a comparable factor.

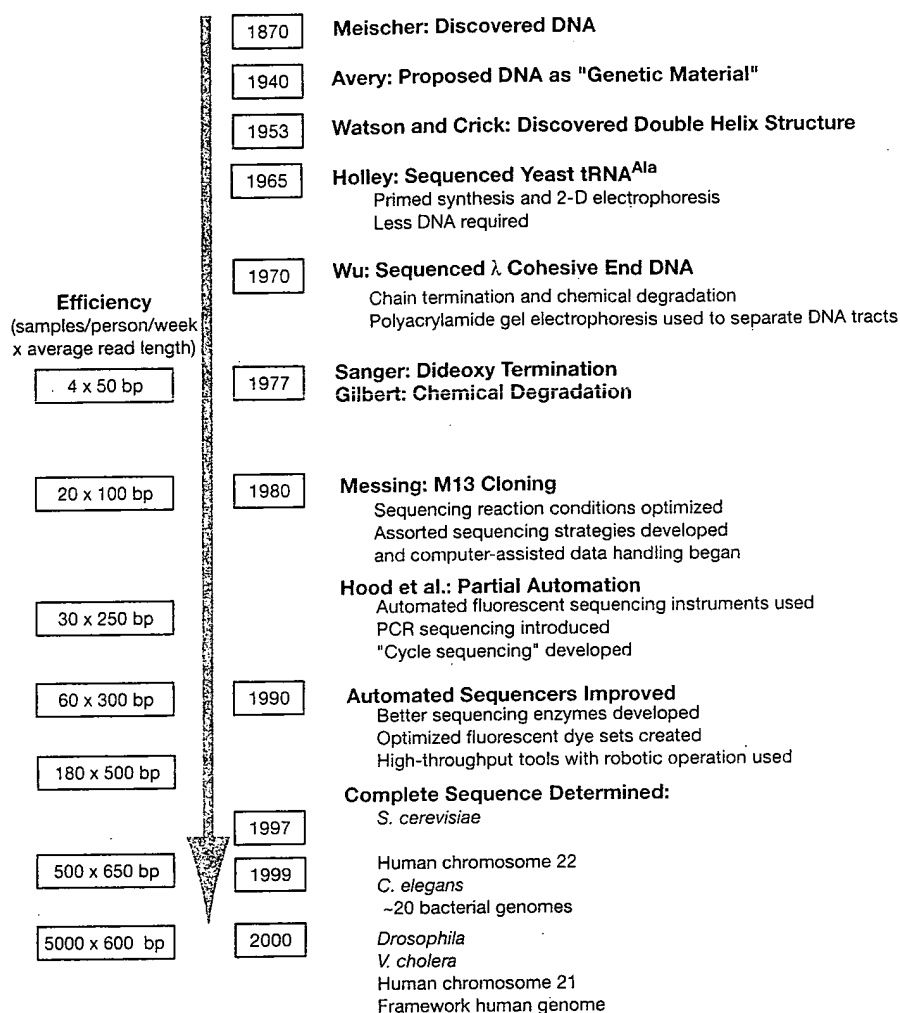


FIGURE 12-10 DNA Sequencing History

Courtesy of E.Y. Chen.

Of course, sequencing efficiency is also directly related to the nature of the DNA substrate. In general practice, DNA molecules that contain more repetitive sequence tracks or homopolymer sequence elements, or higher GC content, require more work (E.Y. Chen et al. 1996). Within a single project, some regions also tend to be more troublesome. To solve specific problems, several modifications of the dideoxy method have been developed in the past decade. For example, the use of nucleotide analogs such as inosine or deaza compounds can help eliminate "gel-compression" problems (Jensen et al. 1991), in which a sequence tract is concealed in a single unresolved band; the inclusion of pyrophosphatase to decompose the pyrophosphate accumulated during chain elongation prevents the loss of peaks ("missing peaks") resulting from reverse polymerase reactions (Tabor and Richardson 1990); the addition of single-strand-binding proteins to sequencing reactions improves the quality of the data produced from DNA templates enriched in looping structures (Chen et al. 1991); and, finally, the use of different cloning vectors provides an alternative when a particular M13 clone is unstable (Chen and Seeburg 1985; Chisoe et al. 1997). Many of these variations have been incorporated into current automated sequencing protocols, particularly as part of extensive projects.

Methods in Automated Sequencing

The following discussion is based on the specifications of the ABI model Automated Sequencer, the equipment currently used by most sequencing facilities.

Dye-Primer Sequencing

In sequencing with dye primers, four separate reactions are carried out for each DNA sample, each reaction containing a different dye-labeled primer. This set of four reactions is then mixed and loaded into a single channel for gel electrophoresis (Smith et al. 1986; Lee et al. 1997). The sequencing reagent "kit" has a set of four primers, each containing a different dye incorporated into an oligonucleotide, typically 17–20 bases in length. Sensitivity can be improved and signal intensity enhanced by using "energy transfer" (ET) primers that carry two separate dyes (Ju et al. 1995; Lee et al. 1997). These ET primers typically contain a single donor dye, which can be easily excited by the argon ion laser. The donor dye transfers the energy to one of four secondary (acceptor) dyes, each with a distinctive emission spectrum. Compared to the earlier single-dye primer sets, the ET primers typically provide three- to fourfold stronger signals (Ju et al. 1995). Amersham and PE Biosystems are two vendors of dye-primer reagents. Amersham (ET primer kit) provides the two dyes attached at two different bases, about five nucleotides apart; PE Biosystems (BigDye-primer kit) provides the two dyes connected by an aminobenzoic acid linker (the so-called BigDye), both attached to the 5' end of the primers.

Either *Taq* polymerase, T7 DNA polymerase (Sequenase; Tabor and Richardson 1987a, 1995), or Thermo Sequenase (Reeve and Fuller 1995) can be used, but the *Taq* polymerase mutant (e.g., AmpliTaq FS from PE Biosystems) is frequently used under cycle sequencing conditions. With thermostable enzymes, the signal is linearly amplified, and hence only 0.1 µg or less of template is needed. This feature is especially important when a robotic operation is used to prepare hundreds of templates in smaller quantities. The standard sequencing kit replaces dGTP with deaza-dGTP to reduce gel compression (see above). For unknown reasons, the use of the deaza compound sometimes results in peak broadening, which reduces the readable length of sequencing tracts. Nevertheless, the average read-length with dye-primer sequencing has now reached 650–700 bp at >99% accuracy in 11–12-hour runs (2400 V with 48-cm slab gel) on the ABI 377 Sequencer (C. Chen et al. 1996). Detailed experimental protocols are provided with convenient manufacturer's kits that contain all of the reagents necessary for dye-primer sequencing reactions.

Dye-Terminator Systems

Although the dye-primer method is suitable for sequencing projects that use universal primers, projects that require custom-designed primers become cumbersome and expensive because each primer must be modified in four separate dye-labeling reactions. One way around this problem is to attach the fluorescent dyes to dideoxynucleotides that become incorporated at the 3' end of the products of sequencing reactions (L. Lee et al. 1992; Rosenblum et al. 1997). Each of the four ddNTPs is labeled with a different dye linked to the nitrogenous base via a linker. Four chain-extension reactions can then be carried out with the same primer in a single tube, sparing considerable labor and cost. By contrast to dye-primers, dye-terminator chemistry has the additional advantage that it eliminates noise arising from premature chain termination without attendant incorporation of dideoxynucleotides.

In its earlier phase of development, dye-terminator chemistry had major problems resulting from the attachment of bulky dye moieties to the dideoxy terminator molecules. Because substrate affinity was altered, it became necessary to tailor a specific set of dye-labeled terminators for use with each DNA polymerase (L. Lee et al. 1992). Unfortunately, the intensity of the signals generated by these polymerase-substrate pairs was frustratingly uneven, which reduced the accuracy of base calling and limited the range of readable sequence (please see Figure 12-11A). During the late 1990s, this problem was largely solved by the use of modified enzymes (please see Figure 12-11B and discussion below) as well as by the introduction of optimized sets of new dyes and linker arms (Figure 12-11C,D). These improvements provided clearer peak patterns, minimal mobility shifts, and cleaner signals. As a consequence, the quality of the data from current dye-terminator sequencing became comparable to results obtained using dye-primers (Rosenblum et al. 1997; Heiner et al. 1998).

At present, two sets of second-generation dye terminators are available. One incorporates dichlororhodamine dyes (dRhodamine Terminator, PE Biosystems); the other incorporates the BigDyes (BigDye Terminator, PE Biosystems). The BigDyes are used in dye-primer sequencing (see above). They contain a fluorescein isomer as the donor dye and four dichlororhodamine dyes as the acceptors. Both terminators work well with AmpliTaq polymerase FS (please see Figure 12-11, bottom panels). Convenient cycle-sequencing conditions are normally used, and the sequencing reactions can be carried out with any primer and with a wide variety of templates (single-stranded DNA, double-stranded DNA, or PCR-generated

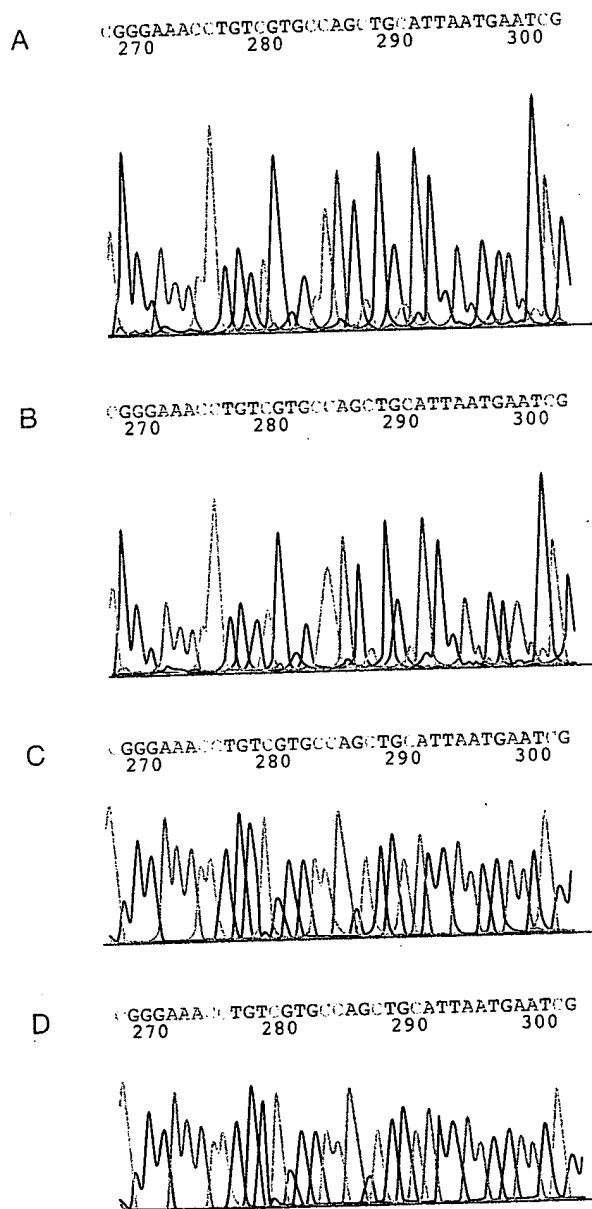


FIGURE 12-11 Comparison of Profiles of Dye-Terminator Sequencing Chemistries

(A) Rhodamine dye terminators sequenced with AmpliTaq polymerase results in very uneven peak pattern. The most noticeable ones are the small C peaks after G, small T peaks after T and G, and large C peaks after C (Parker et al. 1995). (B) Rhodamine dye terminators sequenced with AmpliTaq polymerase FS results in a much improved pattern when compared to the original AmpliTaq sequencing data. However, small G peaks after A and C, and large A peaks after G can sometimes cause errors in base calling (Parker et al. 1996). (C) Dichlororhodamine dye terminators and AmpliTaq polymerase FS result in very even peak patterns. The overall signal is slightly weaker than the rhodamine terminator chemistry, but the weak G peaks after A and large A peaks after G are moderated. (D) BigDye terminators sequenced with AmpliTaq polymerase FS result in very even peak heights and stronger signals. The overall signal and evenness of peak height are much improved compared to dichlororhodamine terminator chemistry. (Courtesy of E.Y. Chen.)

DNA). In addition, because reactions with AmpliTaq FS use far fewer dye terminators, tedious gel-filtration (spin column) steps to remove unincorporated dyes before gel loading can often be replaced by simple ethanol precipitation. Again, dGTP is replaced with dITP (deoxyinosine triphosphate) to reduce gel compressions.

Sequencing Enzymes

Naturally occurring polymerases have features that are often not optimal for DNA sequencing. The relevant and desirable properties of sequencing polymerases include (Tabor and Richardson 1987a, 1989a,b, 1995):

- **High processivity.** Processivity is the degree to which chain extension continues before the enzyme dissociates from a primer-template annealing complex. T7 Sequenase is the most processive of the current catalog of sequencing enzymes, whereas the Klenow fragment is the least.
- **Thermostability.** Resistance to inactivation or dissociation at high temperatures is a most important factor in the cycle-sequencing reactions that are the basis for modern high-throughput sequencing. *Taq* polymerase or variants are the only feasible options.
- **Incorporation of nucleotide analogs such as dye terminators.** The ability to incorporate analogs is a critical factor for the dideoxy chain-termination method. The efficiency of chain termination with each of the dye-labeled terminators must be similar to avoid low-quality data with uneven peaks (see Figure 12-11). Enzymes such as AmpliTaq FS with a high affinity for their dye terminators have an advantage because a much lower concentration of the dye terminators can be used, with savings in both cost and effort otherwise needed to remove the unincorporated dyes.
- **Exonuclease activities.** Polymerases often have 3'-exonuclease "proofreading" and/or 5'-exonuclease activities that remove RNA primers after DNA replication. Because neither activity is desirable for sequencing, variants of the polymerases should be used that lack these activities (e.g., Thermal Sequenase [Amersham] and AmpliTaq polymerase FS [PE Biosystems]).

For more information on modified thermostable DNA polymerases. Please see Table 12-9 in Protocol 5.

Sequencing Templates

Until recently, the primary data in large-scale sequencing operations have been traditionally collected using dye-primer sequencing; dye-terminator sequencing has been used only to fill gaps or to deal with troublesome regions (E.Y. Chen et al. 1996). However, obtaining "finished" data of the highest quality requires that all sequences be acquired either in both orientations or using both chemistries. Comparison of the results obtained using both chemistries provides a check of sequence quality, because the pattern of errors for each chemistry is usually different (Chissoe et al. 1997). With recent improvements, however, the less laborious dye-terminator method is gradually replacing dye-primers in most large-scale sequencing projects. Sequencing with two sets of dye terminators, dRhodamine and BigDye, could become a new standard for the achievement of the desired "double coverage."

BigDye terminator sequencing reactions work well with a wide variety of templates, including single-stranded, double-stranded, and PCR-generated DNAs, as well as with bacterial artificial chromosome (BAC) clones or genomic DNA fragments as large as 5 Mb (C. Chen et al. 1996; Heiner et al. 1998). The optimal sequencing conditions for various templates are given in Table 12-22. Although the intensity of signals tends to be lower with larger templates, base-calling quality remains high, because the baseline noise is sharply reduced by the increased brightness and improved spectral resolution of the dyes (Rosenblum et al. 1997). To compensate for the lower molarity of larger templates in sequencing reactions, increased primer concentrations (2–5x) may be used, and the reaction can be maintained for an increased number of cycles. These alterations enhance signal intensities and produce more accurate, longer read-lengths.

Optimizing the Reaction

Several other factors remain important for achieving optimal results with automated sequencing.

- Although this is a "low-tech" consideration, it is critical to use template DNA clean and free of any contaminants that might interfere with primer annealing. For PCR products in particular, contaminating oligonucleotides and dNTPs can be conveniently digested by *ExoI*/SAP treatment (Hanke and Wink 1994; Werle et al. 1994; C. Chen et al. 1996).
- For loading on capillary-based automated DNA sequencers, samples must be free of ions as they interfere with the electrokinetic injection process. Take special care to eliminate excess dye-terminators before loading samples on gels in both conventional and automated sequencers. This is particularly important when template DNAs are large, since residual dyes can make obscure low-intensity signals (Heiner et al. 1998).

TABLE 12-22 BigDye-Terminator Sequencing Conditions for Various Types of Templates

Template types	M13/ PCR Products	Plasmid	BAC	Microbial DNA
Typical sizes	2–9 kb	<10 kb	200 kb	up to 5 Mb
Reaction mix				
Template DNA (approximately)	30 ng	400 ng	500 ng	2–3 µg
BigDye Terminator Mix	3 µl	4 µl	16 µl	16 µl
Primer	3 pmoles	3 pmoles	6 pmoles	12 pmoles
Total volume	10 µl	10 µl	40 µl	40 µl
Cycling conditions				
Initial denaturation	96°C/1 minute	96°C/1 minute	95°C/5 minutes	95°C/5 minutes
Denaturation	96°C/10 seconds	96°C/10 seconds	95°C/30 seconds	95°C/30 seconds
Annealing	52°C/10 seconds	52°C/10 seconds	55°C/20 seconds	55°C/20 seconds
Extension	60°C/4 minutes	60°C/4 minutes	60°C/4 minutes	60°C/4 minutes
Number of cycles	25	25–50	30–75	45–99
Reaction clean-Up				
Ethanol or isopropanol precipitation	yes	yes	yes	yes?
G-50 Spun column	no	no	yes	yes
Loading on 377 Sequencing Gel				
Resuspension volume	3+ µl	3+ µl	2 µl	2 µl
Loading volume	1 µl	1 µl	1–2 µl	2 µl
Read-length (bases)	750+	750+	550–750	500–600

Courtesy of E.Y. Chen.

- As might be expected, the design of the primer is critical. In general, the same rules are used to design primers for automated sequencers and for sequencing by hand. In brief, successful primers consist of a unique sequence of 16–25 nucleotides (the larger the template DNAs, the longer the oligonucleotides), with an appropriate GC content and a low predisposition to fold into secondary structure (Buck et al. 1999).
- For laboratories analyzing large numbers of samples, an effective quality-controlling system, together with operational tools such as liquid-handling robots, is essential to maintain consistently high-throughput and accuracy.

Genome Sequencing Strategy

The expansive increase in data throughput resulting from technological advances has been accompanied by innovations in sequencing strategies. Until 1998, the general approach taken for sequencing larger genomes (those >5 Mb, including yeast, nematode, *Arabidopsis*, and humans) consisted of mapping followed by sequencing. Specifically, the construction of overlapping arrays of large-insert *E. coli* subclones (e.g., BACs) averaging 150–200 kb was followed by the complete sequencing of each of these clones one by one. The BAC clones are sequenced by a well-established shotgun method that uses random cloning of 1–2-kb fragments, followed by the sequencing of enough samples to cover the target region randomly.

With increased sequencing throughput, the mapping process tends to become a bottleneck. However, recent sequencing approaches based on a whole-genome shotgun approach (Weber and Myers 1997; Venter et al. 1998) have successfully challenged the traditional clone-by-clone strategy (Green 1997). This strategy has been used as an alternative to finish the Human Genome Project and to move ahead with mouse and other complex genomes (for news report, see Pennisi 2000). The technical difference lies in the fact that the whole-genome shotgun approach for a large genome like that of the human requires the “simultaneous” assembly of some 60 million sequence reads (~500 bases each), whereas the former strategy (mapping followed by sequencing) relies on the assembly of a few thousand reads at a time. Thus, intense arguments

among the proponents of the two approaches have focused on issues such as computing capability, gap-filling problems, and cost efficiency. Nevertheless, it is generally agreed that a whole-genome shotgun approach can rapidly generate new data, albeit in an often fragmentary form, to facilitate faster discovery of novel genes. The successful application of this strategy is dependent on high-powered computers and sophisticated software that can digitize data quality while comparing and matching the reads from both ends of each of the millions of sequencing samples. In the real world, shotgun approaches also make use of a variety of mapping information from clones, genes, and genetic markers, all of which help to assist assembly. Furthermore, using DNA samples from multiple individuals will likely result in the discovery and cataloging of millions of polymorphic sites. By early 2000, a growing number of scientists believed that a combination of the map/sequence and shotgun strategies was the optimal approach (see Pennisi 2000).

Prospects

The last 15 years have brought sequencing instrumentation and chemistry to a point that truly approaches fully automatic sequencing (please see Figure 12-10). Accompanying improvements in base-calling software, assembly software, and template preparations have progressively simplified both the sequencing operation and the later editing processes. As a result, the limiting factors for most large-scale projects are the purchase and upkeep of machines and the cost of reagents rather than the involvement of a large number of highly skilled personnel. Investigators should thus be free to carry out the tasks for which automated sequencing was invented in the first place — to identify genes, rivet them to physical maps, chase them to their evolutionary roots, and chart their organization, patterns of expression, and biological function.

MICROTITER PLATES

The components of dideoxy-mediated DNA sequencing reactions can be assembled in the wells of heat-resistant microtiter plates with 96 U-shaped wells of ~300- μ l capacity. All steps of the subsequent reactions — from initial annealing of primers and template to addition of formamide-dye stop mixture — can then be carried out without transferring the reaction mixtures to fresh tubes or plates. Microtiter plates are especially valuable when several sets of sequencing reactions are performed simultaneously. The various components of the reaction can be transferred to the wells with 8- or 12-channel multichannel pipettors so efficiently that 96 sequencing reactions can be completed with ease within 1 hour. Because the individual components of the reactions are delivered as droplets to the walls of the wells, all 96 reactions can be started simultaneously simply by centrifuging the plates in a swing out rotor equipped with plate holders. Both fluorescent and radioactive labels can be accommodated (Smith et al. 1993). The following are the chief problems.

- **Heating the microtiter plate** during the annealing step, so that the fluid in all of the wells of the plate is the same. It is important to use microtiter plates that are designed for use in thermal cyclers and are thin enough to facilitate rapid transfer of heat into and out of the plate. Incubation at 55°C (the annealing step), 37°C (the extension-termination step), or 85°C (the denaturation step) can then be carried out in a thermal cycler or by purchasing or constructing a heating block that can be accommodated in a standard modular heating unit (Koop et al. 1990). Sequencing reactions were heated in earlier days by floating open microtiter plates, like armadas, on the surface of water baths. This was fun but led to contamination of many water baths by radioactivity and the ruination of many sequencing reactions by flooding or evaporation.
- **Condensation and evaporation.** Nowadays most heating devices and thermal cyclers are usually equipped with heated covers, which greatly reduce evaporation and condensation. Evaporation can be further reduced by covering the plate with Saran Wrap or with a well-fitting plastic lid (e.g., Falcon 3913; Becton Dickinson).
- **Cross-contamination of wells.** Careful handling is required to prevent accidental transfer of fluid between different wells. It is best to seal the plates for the mixing and storage steps with plastic plate sealers and pressure-sensitive film (Falcon 3073; Becton Dickinson).
- **Centrifugation.** A centrifuge fitted with a rotor capable of supporting microtiter plates and capable of speeds up to 4000 rpm is required. The rotor trays should be well-padded with rubber or polystyrene foam.

FULL TEXT OF CASES (USPQ2D)
All Other Cases

In re Vaeck (CA FC) 20 USPQ2d 1438 In re Vaeck

**U.S. Court of Appeals Federal Circuit
20 USPQ2d 1438**

**Decided October 21, 1991
No. 91-1120**

Headnotes

PATENTS

1. Patentability/Validity - Obviousness - Combining references (§ 115.0905)

Rejection of claimed subject matter as obvious under 35 USC 103 in view of combination of prior art references requires consideration of whether prior art would have suggested to those of ordinary skill in art that they should make claimed composition or device, or carry out claimed process, and whether prior art would also have revealed that such person would have reasonable expectation of success; both suggestion and reasonable expectation of success must be founded in prior art, not in applicant's disclosure.

2. Patentability/Validity - Obviousness - Relevant prior art - Particular inventions (§ 115.0903.03)

Patent and Trademark Office has failed to establish prima facie obviousness of claims for use of genetic engineering techniques for producing proteins that are toxic to insects such as larvae of mosquitos and black flies, since prior art does not disclose or suggest expression in cyanobacteria of chimeric gene encoding insecticidally active protein, or convey to those of ordinary skill reasonable expectation of success in doing so; expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious expression of unrelated genes in cyanobacteria for unrelated purposes.

3. Patentability/Validity - Specification - Enablement (§ 115.1105)**JUDICIAL PRACTICE AND PROCEDURE****Procedure - Judicial review - Standard of review - Patents (§ 410.4607.09)**

Specification must, in order to be enabling as required by 35 USC 112, first paragraph, teach person skilled in art to make and use invention without "undue experimentation," which does not preclude some experimentation; enablement is question of law which is reviewed independently on appeal, although such determination is based upon underlying factual findings which are reviewed for clear error.

PATENTS**4. Patentability/Validity - Specification - Enablement (§ 115.1105)**

Patent and Trademark Office did not err in rejecting, as non-enabling pursuant to 35 USC 112, first paragraph, claims for use of genetic engineering techniques for producing proteins that are toxic to insects such as larvae of mosquitos and black flies, in view of relatively incomplete understanding of biology of cyanobacteria as of applicants' filing date, as well as limited disclosure by applicants of particular cyanobacterial genera operative in claimed invention, since there is no reasonable correlation between narrow disclosure in applicants' specification and broad scope of protection sought in claims encompassing gene expression in any and all cyanobacteria.

Case History and Disposition:

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Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Application for patent, serial no. 07/021,405, filed March 4, 1987, by Mark A. Vaeck, Wipa Chungjatupornchai, and Lee McIntosh (hybrid genes incorporating a DNA fragment containing a gene coding for an insecticidal protein, plasmids, transformed cyanobacteria expressing such protein and method for use as a biocontrol agent). From decision rejecting claims 1-48 and 50-52 as unpatentable under 35 USC 103, and rejecting claims 1-48 and 50-51 for lack of enablement, applicants appeal. Affirmed and part and reversed in part; Mayer, J., dissents with opinion.

Attorneys:

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Teddy S. Gron, associate solicitor (Fred E. McKelvey, solicitor and Richard E. Schafer, associate solicitor, with him on brief), for appellee.

Judge:

Before Rich, Archer, and Mayer, circuit judges.

Opinion Text

Opinion By:

Rich, J.

This appeal is from the September 12, 1990 decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), affirming the examiner's rejection of claims 1-48 and 50-52 of application Serial No. 07/021,405, filed March 4, 1987, titled "Hybrid Genes Incorporating a DNA Fragment Containing a Gene Coding for an Insecticidal Protein, Plasmids, Transformed Cyanobacteria Expressing Such Protein and Method for Use as a Biocontrol Agent" as unpatentable under 35 USC 103, as well as the rejection of claims 1-48 and 50-51 under 35 USC 112, first paragraph, for lack of enablement. We reverse the § 103 rejection. The § 112 rejection is affirmed in part and reversed in part.

BACKGROUND

A. The Invention

The claimed invention is directed to the use of genetic engineering techniques 1 for production of proteins that are toxic to insects such as larvae of mosquitos and black flies. These swamp-dwelling pests are the source of numerous human health problems, including malaria. It is known that certain species of the naturally-occurring *Bacillus* genus of bacteria produce proteins ("endotoxins") that are toxic to these insects. Prior art methods of combatting the insects involved spreading or spraying crystalline spores of the insecticidal *Bacillus* proteins over swamps. The spores were environmentally unstable, however, and would often sink to the bottom of a swamp before being consumed, thus rendering this method prohibitively expensive. Hence the need for a lower-cost method of producing the insecticidal *Bacillus* proteins in high volume, with application in a more stable vehicle. As described by appellants, the claimed subject matter meets this need by providing for the production of the insecticidal *Bacillus* proteins within host cyanobacteria. Although both cyanobacteria and bacteria are members of the procaryote 2 kingdom, the

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cyanobacteria (which in the past have been referred to as "blue-green algae") are unique among procaryotes in that the cyanobacteria are capable of oxygenic photosynthesis. The cyanobacteria grow on top of swamps where they are consumed by mosquitos and black flies. Thus, when *Bacillus* proteins are produced within transformed 3 cyanobacterial hosts according to the claimed invention, the presence of the insecticide in the food of the targeted insects advantageously guarantees direct uptake by the insects.

More particularly, the subject matter of the application on appeal includes a chimeric (i.e., hybrid) gene comprising (1) a gene derived from a bacterium of the *Bacillus* genus whose product is an insecticidal protein, united with (2)

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a DNA promoter effective for expressing 4 the *Bacillus* gene in a host cyanobacterium, so as to produce the desired insecticidal protein.

The claims on appeal are 1-48 and 50-52, all claims remaining in the application. Claim 1 reads:

1. A chimeric gene capable of being expressed in Cyanobacteria cells comprising:

(a) a DNA fragment comprising a promoter region which is effective for expression of a DNA fragment in a Cyanobacterium; and

(b) at least one DNA fragment coding for an insecticidally active protein produced by a *Bacillus* strain, or coding for an insecticidally active truncated form of the above protein or coding for a protein having substantial sequence homology to the active protein,

the DNA fragments being linked so that the gene is expressed.

Claims 2-15, which depend from claim 1, recite preferred *Bacillus* species, promoters, and selectable markers. 5 Independent claim 16 and claims 17-31 which depend therefrom are directed to a hybrid plasmid vector which includes the chimeric gene of claim 1. Claim 32 recites a bacterial strain. Independent claim 33 and claims 34-48 which depend therefrom recite a cyanobacterium which expresses the chimeric gene of claim 1. Claims 50-51 recite an insecticidal composition. Claim 52 recites a particular plasmid that appellants have deposited.

B. Appellants' Disclosure

In addition to describing the claimed invention in generic terms, appellants' specification discloses two particular species of *Bacillus* (*B. thuringiensis*, *B. sphaericus*) as sources of insecticidal protein; and nine genera of cyanobacteria (*Synechocystis*, *Anacystis*, *Synechococcus*, *Agmenellum*, *Aphanocapsa*, *Gloecapsa*, *Nostoc*, *Anabaena* and *Ffremyllia*) as useful hosts.

The working examples relevant to the claims on appeal detail the transformation of a single strain of cyanobacteria, i.e., *Synechocystis* 6803. In one example, *Synechocystis* 6803 cells are transformed with a plasmid comprising (1) a gene encoding a particular insecticidal protein ("B.t. 8") from *Bacillus thuringiensis* var. *israelensis*, linked to (2) a particular promoter, the P_L promoter from the bacteriophage Lambda (a virus of *E. coli*). In another example, a different promoter, i.e., the *Synechocystis* 6803 promoter for the rubisco operon, is utilized instead of the Lambda P_L promoter.

C. The Prior Art

A total of eleven prior art references were cited and applied, in various combinations, against the claims on appeal. The focus of Dzelzkalns, 6 the primary reference cited against all of the rejected claims, is to determine whether chloroplast promoter sequences can function in cyanobacteria. To that end Dzelzkalns discloses the expression in cyanobacteria of a chimeric gene comprising a chloroplast promoter sequence fused to a gene encoding the enzyme chloramphenicol acetyl transferase (CAT). 7 Importantly, Dzelzkalns teaches the use of the CAT gene as a "marker" gene; this use of antibiotic resistance-conferring genes for selection purposes is a common technique in genetic engineering.

Sekar I, 8 Sekar II, 9 and Ganesan 10 collectively disclose expression of genes encoding certain *Bacillus* insecticidal proteins in the bacterial hosts *B. megaterium*, *B. subtilis* and *E. coli*.

Friedberg 11 discloses the transformation of the cyanobacterium *Anacystis nidulans* R2 by a plasmid vector comprising the O_LP_L operator-promoter region and a temperature-sensitive repressor gene of the bacteriophage Lambda. While the cyanobacteria are attractive organisms for the cloning of genes involved in photosynthesis, Friedberg states, problems may still be encountered such as suboptimal expression of the cloned gene, detrimental effects on cell growth of overexpressed, highly hydrophobic proteins, and rapid turnover of some gene products.

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To address these problems, Friedberg teaches the use of the disclosed Lambda regulatory signals in plasmid vehicles which, it states, have "considerable potential for use as vectors the expression of which can be controlled in *Anacystis*"

Miller 12 compares the initiation specificities *in vitro* of DNA-dependent RNA polymerases 13 purified from two different species of cyanobacteria (*Fremyella diplosiphon* and *Anacystis nidulans*), as well as from *E. coli*. Nierzwicki-Bauer 14 identifies in the cyanobacterium *Anabaena* 7120 the start site for transcription of the gene encoding *rbc* L, the large subunit of the enzyme ribulose-1, 5-bisphosphate carboxylase. It reports that the nucleotide sequence 14-8 base pairs preceding the transcription start site "resembles a good *Escherichia coli* promoter," but that the sequence 35 base pairs before the start site does not.

Chauvat 15 discloses host-vector systems for gene cloning in the cyanobacterium *Synechocystis* 6803, in which the antibiotic resistance-conferring *neo* gene is utilized as a selectable marker.

Reiss 16 studies expression in *E. coli* of various proteins formed by fusion of certain foreign DNA sequences with the *neo* gene.

Kolowsky 17 discloses chimeric plasmids designed for transformation of the cyanobacterium *Synechococcus* R2, comprising an antibiotic-resistant gene linked to chromosomal DNA from the *Synechococcus* cyanobacterium.

Barnes, United States Patent No. 4,695,455, is directed to the treatment with stabilizing chemical reagents of pesticides produced by expression of heterologous genes (such as those encoding *Bacillus* proteins) in host microbial cells such as *Pseudomonas* bacteria. The host cells are killed by this treatment, but the resulting pesticidal compositions exhibit prolonged toxic activity when exposed to the environment of target pests.

D. The Grounds of Rejection

1. The § 103 Rejections

Claims 1-6, 16-21, 33-38, 47-48 and 52 (which include all independent claims in the application) were rejected as unpatentable under 35 USC 103 based upon Dzelzkalns in view of Sekar I or Sekar II and Ganesan. The examiner stated that Dzelzkalns discloses a chimeric gene capable of being highly expressed in a cyanobacterium, said gene comprising a promoter region effective for expression in a cyanobacterium operably linked to a structural gene encoding CAT. The examiner acknowledged that the chimeric gene and transformed host of Dzelzkalns differ from the claimed invention in that the former's structural gene encodes CAT rather than insecticidally active protein. However, the examiner pointed out, Sekar I, Sekar II, and Ganesan teach genes encoding insecticidally active proteins produced by *Bacillus*, and the advantages of expressing such genes in heterologous 18 hosts to obtain larger quantities of the protein. The examiner contended that it would have been obvious to one of ordinary skill in the art to substitute the *Bacillus* genes taught by Sekar I, Sekar II, and Ganesan for the CAT gene in the vectors of Dzelzkalns in order to obtain high level expression of the *Bacillus* genes in the transformed cyanobacteria. The examiner further contended that it would have been obvious to use cyanobacteria as heterologous hosts for expression of the claimed genes due to the ability of cyanobacteria to serve as transformed hosts for the

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expression of heterologous genes. In the absence of evidence to the contrary, the examiner contended, the invention as a whole was *prima facie* obvious.

Additional rejections were entered against various groups of dependent claims which we need not address here.

All additional rejections were made in view of Dzelzkalns in combination with Sekar I, Sekar II, and Ganesan, and further in view of other references discussed in Part C above.

The Board affirmed the § 103 rejections, basically adopting the examiner's Answer as its opinion while adding a few comments. The legal conclusion of obviousness does not require absolute certainty, the Board added, but only

a reasonable expectation of success, citing *In re O'Farrell*, 853 F.2d 894, 7 USPQ2d 1673 (Fed. Cir. 1988). In view of the disclosures of the prior art, the Board concluded, one of ordinary skill in the art would have been motivated by a reasonable expectation of success to make the substitution suggested by the examiner.

2. The § 112 Rejection

The examiner also rejected claims 1-48 and 50-51 under 35 USC 112, first paragraph, on the ground that the disclosure was enabling only for claims limited in accordance with the specification as filed. Citing *Manual of Patent Examining Procedure* (MPEP) provisions 706.03(n) 19 and (z) 20 as support, the examiner took the position that undue experimentation would be required of the art worker to practice the claimed invention, in view of the unpredictability in the art, the breadth of the claims, the limited number of working examples and the limited guidance provided in the specification. With respect to unpredictability, the examiner stated that the cyanobacteria comprise a large and diverse group of photosynthetic bacteria including large numbers of species in some 150 different genera including *Synechocystis*, *Anacystis*, *Synechococcus*, *Agmenellum*, *Nostoc*, *Anabaena*, etc. The molecular biology of these organisms has only recently become the subject of intensive investigation and this work is limited to a few genera. Therefore the level of unpredictability regarding heterologous gene expression in this large, diverse and relatively poorly studied group of procaryotes is high....

The Board affirmed, noting that "the limited guidance in the specification, considered in light of the relatively high degree of unpredictability in this particular art, would not have enabled one having ordinary skill in the art to practice the broad scope of the claimed invention without undue experimentation. *In re Fisher*, 427 F.2d 833, 166 USPQ 18 (CCPA 1970)."

OPINION

A. Obviousness

We first address whether the PTO erred in rejecting the claims on appeal as prima facie obvious within the meaning of 35 USC 103. Obviousness is a legal question which this court independently reviews, though based upon underlying factual findings which we review under the clearly erroneous standard. *In re Woodruff*, 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990).

[1] Where claimed subject matter has been rejected as obvious in view of a combination of prior art references, a proper analysis under § 103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. See *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure. *Id.*

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[2] We agree with appellants that the PTO has not established the prima facie obviousness of the claimed subject matter. The prior art simply does not disclose or suggest the expression in cyanobacteria of a chimeric gene encoding an insecticidally active protein, or convey to those of ordinary skill a reasonable expectation of success in doing so. More particularly, there is no suggestion in Dzelzkalns, the primary reference cited against all claims, of substituting in the disclosed plasmid a structural gene encoding *Bacillus* insecticidal proteins for the CAT gene utilized for selection purposes. The expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious the expression of unrelated genes in cyanobacteria for unrelated purposes.

The PTO argues that the substitution of insecticidal *Bacillus* genes for CAT marker genes in cyanobacteria is suggested by the secondary references Sekar I, Sekar II, and Ganesan, which collectively disclose expression of

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genes encoding *Bacillus* insecticidal proteins in two species of host *Bacillus* bacteria (*B. megaterium* and *B. subtilis*) as well as in the bacterium *E. coli*. While these references disclose expression of *Bacillus* genes encoding insecticidal proteins in certain transformed *bacterial* hosts, nowhere do these references disclose or suggest expression of such genes in transformed *cyanobacterial* hosts.

To remedy this deficiency, the PTO emphasizes similarity between bacteria and cyanobacteria, namely, that these are both procaryotic organisms, and argues that this fact would suggest to those of ordinary skill the use of cyanobacteria as hosts for expression of the claimed chimeric genes. While it is true that bacteria and cyanobacteria are now both classified as procaryotes, that fact alone is not sufficient to motivate the art worker as the PTO contends. As the PTO concedes, cyanobacteria and bacteria are not identical; they are classified as two separate divisions of the kingdom Procaryotae. 21 Moreover, it is only in recent years that the biology of cyanobacteria has been clarified, as evidenced by references in the prior art to "blue-green algae." Such evidence of recent uncertainty regarding the biology of cyanobacteria tends to rebut, rather than support, the PTO's position that one would consider the cyanobacteria effectively interchangeable with bacteria as hosts for expression of the claimed gene.

At oral argument the PTO referred to additional secondary references, not cited against any independent claim (i.e., Friedberg, Miller, and Nierzwicki-Bauer), which it contended disclose certain amino acid sequence homology between bacteria and cyanobacteria. The PTO argued that such homology is a further suggestion to one of ordinary skill to attempt the claimed invention. We disagree. As with the Dzelzkalns, Sekar I, Sekar II, and Ganesan references discussed above, none of these additional references disclose or suggest that cyanobacteria could serve as hosts for expression of genes encoding *Bacillus* insecticidal proteins. In fact, these additional references suggest as much about *differences* between cyanobacteria and bacteria as they do about similarities. For example, Nierzwicki-Bauer reports that a certain nucleotide sequence (i.e., the -10 consensus sequence) in a particular cyanobacterium resembles an *E. coli* promoter, but that another nearby nucleotide sequence (the -35 region) does not. While Miller speaks of certain promoters of the bacteriophage Lambda that are recognized by both cyanobacterial and *E. coli* RNA polymerases, it also discloses that these promoters exhibited differing strengths when exposed to the different polymerases. Differing sensitivities of the respective polymerases to an inhibitor are also disclosed, suggesting differences in the structures of the initiation complexes.

The PTO asks us to agree that the prior art would lead those of ordinary skill to conclude that cyanobacteria are attractive hosts for expression of any and all heterologous genes. Again, we can not. The relevant prior art does indicate that cyanobacteria are attractive hosts for expression of both native and heterologous *genes involved in photosynthesis* (not surprisingly, for the capability of undergoing oxygenic photosynthesis is what makes the cyanobacteria unique among procaryotes). However, these references do not suggest that cyanobacteria would be equally attractive hosts for expression of *unrelated* heterologous genes, such as the claimed genes encoding *Bacillus* insecticidal proteins.

In *O'Farrell*, this court affirmed an obviousness rejection of a claim to a method for

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producing a "predetermined protein in a stable form" in a transformed bacterial host. 853 F.2d at 895, 7 USPQ2d at 1674. The cited references included a prior art publication (the Polisky reference) whose three authors included two of the three coinventor-appellants. The main difference between the prior art and the claim at issue was that in Polisky, the heterologous gene was a gene for ribosomal RNA, while the claimed invention substituted a gene coding for a predetermined protein. *Id.* at 901, 7 USPQ2d at 1679. Although, as the appellants therein pointed out, the ribosomal RNA gene is not normally translated into protein, Polisky mentioned preliminary evidence that the transcript of the ribosomal RNA gene was translated into protein, and further predicted that if a gene coding for a protein were to be substituted, extensive translation might result. *Id.* We thus affirmed, explaining that the prior art explicitly suggested the substitution that is the difference between the claimed invention and the prior

art, and presented preliminary evidence suggesting that the [claimed] method could be used to make proteins.

....

... Polisky contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful.

Id. at 901-02, 7 USPQ2d at 1679-80.

In contrast with the situation in *O'Farrell*, the prior art in this case offers no suggestion, explicit or implicit, of the substitution that is the difference between the claimed invention and the prior art. Moreover, the "reasonable expectation of success" that was present in *O'Farrell* is not present here. Accordingly, we reverse the § 103 rejections.

B. Enablement

[3] The first paragraph of 35 USC 112 requires, *inter alia*, that the specification of a patent enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation." *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That *some* experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." *Id.* at 736-37, 8 USPQ2d at 1404. Enablement, like obviousness, is a question of law which we independently review, although based upon underlying factual findings which we review for clear error. *See id.* at 735, 8 USPQ2d at 1402.

In response to the § 112 rejection, appellants assert that their invention is "pioneering," and that this should entitle them to claims of broad scope. Narrower claims would provide no real protection, appellants argue, because the level of skill in this art is so high, art workers could easily avoid the claims. Given the disclosure in their specification, appellants contend that any skilled microbiologist could construct vectors and transform many different cyanobacteria, using a variety of promoters and *Bacillus* DNA, and could easily determine whether or not the active *Bacillus* protein was successfully expressed by the cyanobacteria.

The PTO made no finding on whether the claimed invention is indeed "pioneering," and we need not address the issue here. With the exception of claims 47 and 48, the claims rejected under § 112 are not limited to any particular genus or species of cyanobacteria. The PTO's position is that the cyanobacteria are a diverse and relatively poorly studied group of organisms, comprising some 150 different genera, and that heterologous gene expression in cyanobacteria is "unpredictable." Appellants have not effectively disputed these assertions. Moreover, we note that only one particular species of cyanobacteria is employed in the working examples of appellants' specification, and only nine genera of cyanobacteria are mentioned in the entire document.

[4] Taking into account the relatively incomplete understanding of the biology of cyanobacteria as of appellants' filing date, as well as the limited disclosure by appellants of particular cyanobacterial genera operative in the claimed invention, we are not persuaded that the PTO erred in rejecting claims 1-46 and 50-51 under § 112, first paragraph. There is no reasonable correlation between the narrow disclosure in appellants' specification and the broad scope of protection sought in the claims encompassing gene expression in any and all cyanobacteria. *See In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (the first paragraph of § 112 requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification).

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22 Accordingly, we affirm the § 112 rejection as to those claims.

In so doing we do *not* imply that patent applicants in art areas currently denominated as "unpredictable" must never be allowed generic claims encompassing more than the particular species disclosed in their specification. It is well settled that patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art. *In re Angstadt*, 537 F.2d 498, 502-03, 190 USPQ 214, 218 (CCPA 1976). However, there

must be sufficient disclosure, either through illustrative examples or terminology, 23 to teach those of ordinary skill how to make and how to use the invention as broadly as it is claimed. This means that the disclosure must adequately guide the art worker to determine, without undue experimentation, which species among all those encompassed by the claimed genus possess the disclosed utility. Where, as here, a claimed genus represents a diverse and relatively poorly understood group of microorganisms, the required level of disclosure will be greater than, for example, the disclosure of an invention involving a "predictable" factor such as a mechanical or electrical element. *See Fisher*, 427 F.2d at 839, 166 USPQ at 24. In this case, we agree with the PTO that appellants' limited disclosure does not enable one of ordinary skill to make and use the invention as now recited in claims 1-46 and 50-51 without undue experimentation.

Remaining dependent claim 47 recites a cyanobacterium which expresses the chimeric gene of claim 1, wherein the cyanobacterium is selected from among the genera *Anacystis* and *Synechocystis*. Claim 48, which depend from claim 47, is limited to the cyanobacterium *Synechocystis* 6803. The PTO did not separately address these claims, nor indicate why they should be treated in the same manner as the claims encompassing all types of cyanobacteria. Although these claims are not limited to expression of genes encoding particular *Bacillus* proteins, we note what appears to be an extensive understanding in the prior art of the numerous *Bacillus* proteins having toxicity to various insects. The rejection of claims 47-48 under § 112 will not be sustained.

CONCLUSION

The rejection of claims 1-48 and 50-52 under 35 USC 103 is *reversed*. The rejection of claims 1-46 and 50-51 under 35 USC 112, first paragraph, is *affirmed* and the rejection of claims 47 and 48 thereunder is *reversed*.
AFFIRMED-IN-PART, REVERSED-IN-PART

Footnotes

Footnote 1. Basic vocabulary and techniques for gene cloning and expression have been described in *In re O'Farrell*, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988), and are not repeated here.

Footnote 2. All living cells can be classified into one of two broad groups, procaryotes and eucaryotes. The procaryotes comprise organisms formed of cells that do not have a distinct nucleus; their DNA floats throughout the cellular cytoplasm. In contrast, the cells of eucaryotic organisms such as man, other animals, plants, protozoa, algae and yeast have a distinct nucleus wherein their DNA resides.

Footnote 3. "Transformed" cyanobacteria are those that have successfully taken up the foreign *Bacillus* DNA such that the DNA information has become a permanent part of the host cyanobacteria, to be replicated as new cyanobacteria are generated.

Footnote 4. "Expression" of a gene refers to the production of the protein which the gene encodes; more specifically, it is the process of transferring information from a gene (which consists of DNA) via messenger RNA to ribosomes where a specific protein is made.

Footnote 5. In the context of the claimed invention, "selectable markers" or "marker genes" refer to antibiotic-resistance conferring DNA fragments, attached to the gene being expressed, which facilitate the selection of successfully transformed cyanobacteria.

Footnote 6. *Nucleic Acids Res.* 8917 (1984).

Footnote 7. Chloramphenicol is an antibiotic; CAT is an enzyme which destroys chloramphenicol and thus imparts resistance thereto.

Footnote 8. *Biochem. and Biophys. Res. Comm.* 748 (1986).

Footnote 9. *Gene* 151 (1985).

Footnote 10. *Mol. Gen. Genet.* 181 (1983).

Footnote 11. *Mol. Gen. Genet.* 505 (1986).

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Footnote 12. *J. Bacteriology* 246 (1979).

Footnote 13. RNA polymerase, the enzyme responsible for making RNA from DNA, binds at specific nucleotide sequences (promoters) in front of genes in DNA, and then moves through the gene making an RNA molecule that includes the information contained in the gene. Initiation specificity is the ability of the RNA polymerase to initiate this process specifically at a site(s) on the DNA template.

Footnote 14. *Proc. Natl. Acad. Sci. USA* 5961 (1984).

Footnote 15. *Mol. Gen. Genet.* 185 (1986).

Footnote 16. *Gene* 211 (1984).

Footnote 17. *Gene* 289 (1984).

Footnote 18. Denotes different species or organism.

Footnote 19. MPEP 706.03(n), "Correspondence of Claim and Disclosure," provides in part:

In chemical cases, a claim may be so broad as to not be supported by [the] disclosure, in which case it is rejected as unwarranted by the disclosure....

Footnote 20. MPEP 796.03(z), "Undue Breadth," provides in part:

In applications directed to inventions in arts where the results are unpredictable, the disclosure of a single species usually does not provide an adequate basis to support generic claims. *In re Sol*, 1938 C.D. 723; 497 O.G. 546. This is because in arts such as chemistry it is not obvious from the disclosure of one species, what other species will work. *In re Dreshfield*, 1940 C.D. 351; 518 O.G. 255 gives this general rule: "It is well settled that in cases involving chemicals and chemical compounds, which differ radically in their properties it must appear in an applicant's specification either by the enumeration of a sufficient number of the members of a group or by other appropriate language, that the chemicals or chemical combinations included in the claims are capable of accomplishing the desired result." ...

Footnote 21. *Stedman's Medical Dictionary* 1139 (24th ed. 1982) (definition of "Procaryotae"). Procaryotic organisms are commonly classified according to the following taxonomic hierarchy: Kingdom; Division; Class; Order; Family; Genus; Species. 3 *Bergey's Manual of Systematic Bacteriology* 1601 (1989).

Footnote 22. The enablement rejection in this case was not based upon a post-filing date state of the art, as in *In re Hogan*, 559 F.2d 595, 605-07, 194 USPQ 527, 536-38 (CCPA 1977). See also *United States Steel Corp. v. Phillips Petroleum Co.*, 865 F.2d 1247, 1251, 9 USPQ2d 1461, 1464 (Fed. Cir. 1989) (citing *Hogan*); *Hormone Research Found., Inc. v. Genentech, Inc.*, 904 F.2d 1558, 1568-69, 15 USPQ2d 1039, 1047-48 (Fed. Cir. 1990) (directing district court, on remand, to consider effect of *Hogan* and *United States Steel* on the enablement analysis of *Fisher*), *cert. dismissed*, — U.S. —, 111 S. Ct. 1434 (1991). We therefore do not consider the effect of *Hogan* and its progeny on *Fisher*'s analysis of when an inventor should be allowed to "dominate the future patentable inventions of others." *Fisher*, 427 F.2d at 839, 166 USPQ at 24.

Footnote 23. The first paragraph of § 112 requires nothing more than *objective* enablement. *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is irrelevant. *Id.*

Dissenting Opinion Text

Dissent By:

Mayer, J., dissenting.

An appeal is not a second opportunity to try a case or prosecute a patent application, and we should not allow parties to "undertake to retry the entire case on appeal." *Perini America, Inc. v. Paper Converting Machine Co.*, 832 F.2d 581, 584, 4 USPQ2d 1621, 1624 (Fed. Cir. 1987); *Eaton Corp. v. Appliance Valves Corp.*, 790 F.2d 874, 877, 229 USPQ 668, 671 (Fed. Cir. 1986). But that is precisely what the court has permitted here. The PTO conducted a thorough examination of the prior art surrounding this patent application and concluded the claims

would have been obvious. The board's decision based on the examiner's answer which comprehensively explains the rejection is persuasive and shows how the evidence supports the legal conclusion that the claims would have been obvious. Yet, the court ignores all this and conducts its own examination, if you will, as though the examiner and board did not exist. Even if thought this opinion were more persuasive than the board's, I could not join it because it misperceives the role of the court.

The scope and content of the prior art, the similarity between the prior art and the claims, the level of ordinary skill in the art, and what the prior art teaches are all questions of fact. *Graham v. John Deere Co.*, 383 U.S. 1, 17, 148 USPQ 459, 467 (1966); *Jurgens v. McKasy*, 927 F.2d 1552, 1560, 18 USPQ2d 1031, 1037 (Fed. Cir. 1991). And "[w]here there are two permissible views of

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the evidence, the factfinder's choice between them cannot be clearly erroneous." *Anderson v. City of Bessemer City*, 470 U.S. 564, 574 (1985). The mere denomination of obviousness as a question of law does not give the court license to decide the factual matters afresh and ignore the requirement that they be respected unless clearly erroneous. *In re Woodruff*, 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990); *In re Kulling*, 897 F.2d 1147, 1149, 14 USPQ2d 1056, 1057 (Fed. Cir. 1990). There may be more than one way to look at the prior art, but on this record we are bound by the PTO's interpretation of the evidence because it is not clearly erroneous and its conclusion is unassailable. I would affirm on that basis.

- End of Case -

FULL TEXT OF CASES (USPQ2D)

All Other Cases

**Amgen Inc. v. Chugai Pharmaceutical Co. Ltd. (CA FC) 18 USPQ2d
1016 Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.**

**U.S. Court of Appeals Federal Circuit
18 USPQ2d 1016**

Decided March 5, 1991

Nos. 90-1273, -1275

Headnotes

PATENTS

1. Patentability/Validity - Date of invention - Conception (§ 115.0403)

Conception of chemical compound requires that inventor be able to define compound so as to distinguish it from other materials, and to describe how to obtain it, rather than simply defining it solely by its principal biological property; thus, when inventor of gene, which is chemical compound albeit complex one, is unable to envision detailed constitution of gene so as to distinguish it from other materials, as well as method for obtaining it, conception is not achieved until reduction to practice has occurred, and until after gene has been isolated.

2. Patentability/Validity - Date of invention - Conception (§ 115.0403)

Conception of generalized approach for screening DNA library that might be used to identify and clone erythropoietin gene of then-unknown constitution is not conception of "purified and isolated DNA sequence" encoding human EPA, since it is not "definite and permanent idea of the complete and operative invention."

3. Patentability/Validity - Obviousness - Relevant prior art - Particular inventions (§ 115.0903.03)

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Federal district court did not err in holding non-obvious claims for purified and isolated DNA sequence encoding human hormone erythropoietin, in view of evidence showing that procedures may have been obvious to try, but also showing that there was no reasonable expectation of success.

4. Patentability/Validity - Specification - Best mode (§ 115.1107)

Determination of whether best mode requirement is satisfied is question of fact and thus is reviewed under clearly erroneous standard.

5. Patentability/Validity - Specification - Best mode (§ 115.1107)

Biological deposit is required to satisfy best mode requirement, for patents involving novel, genetically-engineered biological subject matter, if invention is incapable of being practiced without access to that organism, but if organism is created by insertion of genetic material into cell obtained from generally available sources, then cell deposit itself is not necessary and all that is required is description of best mode and adequate description of means of carrying out invention; if cells can be prepared without undue experimentation from known materials, based on description in patent specification, deposit is not required.

6. Patentability/Validity - Specification - Best mode (§ 115.1107)

Evidence showing that scientists were unable to duplicate inventor's genetically-heterogeneous best mode cell strain does not demonstrate that best mode requirement is not satisfied, since issue is whether disclosure is "adequate," and exact duplication is not necessary.

7. Patentability/Validity - Specification - Enablement (§ 115.1105)

Issue of whether claimed invention is enabled under 35 USC 112 is question of law that is reviewed de novo.

8. Patentability/Validity - Specification - Enablement (§ 115.1105)

Patent applicant is entitled to claim invention generically, if invention is described sufficiently to meet requirements of 35 USC 112; however, applicant, in claims for DNA sequences encoding erythropoietin, which has claimed every possible analog of gene containing about 4,000 nucleotides, but which has provided details for preparing only few EPO analog genes has not provided sufficient disclosure to support its claims, since, in view of structural complexity of EPO gene, manifold possibilities for change in its structure, and uncertainty as to what utility will be possessed by these analogs, additional disclosure is needed as to identifying various analogs within scope of claim, methods for making them, and structural requirements for producing compounds with EPO-like activity.

9. Infringement - Defenses - Fraud or unclean hands (§ 120.1111)

Ultimate conclusion of inequitable conduct is reviewed under abuse of discretion standard, but underlying factual findings are reviewed under clearly erroneous standard.

10. Patentability/Validity - Specification - Enablement (§ 115.1105)

Federal district court erred by concluding that patent for method for purification of erythropoietin sufficiently enabled person of ordinary skill in art to obtain homogeneous EPO from natural sources having mean in vivo specific activity of at least 160,000, since court erred in accepting in vitro data as support for claims containing in vivo limitation.

11. Patentability/Validity - Specification - Claim adequacy (§ 115.1109)**Patent construction - Claims - Defining terms (§ 125.1305)**

Claim whose meaning is in doubt is properly declared invalid, especially when there is close prior art; thus, federal district court did not err in holding that claim for homogeneous erythropoietin which has specific activity limitation of "at least about" 160,000 was indefinite, although such holding does not preclude any and all uses of term "about" in patent claims, since such term may be acceptable in appropriate fact situations.

Particular patents - Chemical - Erythropoietin

4,677,195, Hewick and Seehre, method for the purification of erythropoietin and erythropoietin compositions, claims 1, 3, 4, and 6 invalid.

4,703,008, Lin, DNA sequences encoding erythropoietin, claims 2, 4, and 6 valid and infringed; claims 7, 8, 23-27, and 29 invalid.

Case History and Disposition:

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Appeal from the U.S. District Court for the District of Massachusetts, Young, J. (Saris, U.S. magistrate); 13 USPQ2d 1737 .

Action by Amgen Inc. against Chugai Pharmaceutical Co. Ltd. and Genetics Institute Inc. for infringement of patent no. 4,703,008, to which defendants counterclaimed alleging infringement of patent no. 4,677,195. From federal district court decision holding certain claims of both patents valid and infringed, and holding other claims invalid, parties cross-appeal. Affirmed in part, reversed in part, and vacated in part.

Attorneys:

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Kurt E. Richter, Eugene Moroz, William S. Feiler, and Michael P. Dougherty, of Morgan & Finnegan, New York, N.Y., for Chugai Pharmaceutical.

William F. Lee, William McElwain, Ian Crawford, David Marder, David B. Bassett, and Sarianna T. Honkola, of Hale & Dorr, Boston, Mass., for Genetics Institute.

Judge:

Before Markey, Lourie, and Clevenger, circuit judges.

Opinion Text

Opinion By:

Lourie, J.

This appeal and cross appeal are from the March 4, 1990, judgment of the United States District Court for the District of Massachusetts, No. 87-2617-Y, *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 13 USPQ2d 1737 (1990), and involve issues of patent validity, infringement, and inequitable conduct with respect to two patents: U.S. Patent, 4,703,008 ('008), owned by Kirin-Amgen Inc. (Amgen), and U.S. Patent 4,677,195 ('195), owned by Genetics Institute, Inc. (GI).

Chugai Pharmaceutical Co., Ltd. (Chugai) and Genetics Institute, Inc. (collectively defendants) assert on appeal that the district court erred in holding that: 1) Amgen's '008 patent is not invalid under 35 U.S.C. §§102(g) and 103; 2) the '008 patent is enforceable; 3) the failure of Amgen to deposit the best mode host cells was not a violation of the best mode requirement under 35 U.S.C. §112; and 4) claims 4 and 6 of GI's '195 patent are invalid for indefiniteness under 35 U.S.C. §112.

On cross appeal, Amgen challenges the district court's holdings that: 1) claims 1 and 3 of the '195 patent are enabled; 2) the '195 patent is enforceable; 3) this is not an exceptional case warranting an award of attorney fees to Amgen; and 4) claims 7, 8, 23-27 and 29 of the '008 patent are not enabled by the specification.

We affirm the district court's holdings in all respects, except that we reverse the court's ruling that claims 1 and 3 of the '195 patent are enabled. We also vacate that part of the district court's judgment relating to infringement of those claims.

BACKGROUND 1

Erythropoietin (EPO) is a protein consisting of 165 amino acids which stimulates the production of red blood cells. It is therefore a useful therapeutic agent in the treatment of anemias or blood disorders characterized by low or defective bone marrow production of red blood cells.

The preparation of EPO products generally has been accomplished through the concentration and purification of urine from both healthy individuals and those exhibiting high EPO levels. A new technique for producing EPO is recombinant DNA technology in which EPO is produced from cell cultures into which genetically-engineered vectors containing the EPO gene have been introduced. The production of EPO by recombinant technology involves expressing an EPO gene through the same processes that occur in a natural cell.

THE PATENTS

On June 30, 1987, the United States Patent and Trademark Office (PTO) issued to Dr. Rodney Hewick U.S. Patent 4,677,195, entitled "Method for the Purification of Erythropoietin and Erythropoietin Compositions" (the '195

patent). The patent claims both homogeneous EPO and compositions thereof and a method for purifying human EPO using reverse phase high performance liquid chromatography. The method claims are not before us. The relevant claims of the '195 patent are:

1. Homogeneous erythropoietin characterized by a molecular weight of about 34,000 daltons on SDS PAGE, movement

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as a single peak on reverse phase high performance liquid chromatography and a specific activity of at least 160,000 IU per absorbance unit at 280 nanometers.

3. A pharmaceutical composition for the treatment of anemia comprising a therapeutically effective amount of the homogeneous erythropoietin of claim 1 in a pharmaceutically acceptable vehicle.

4. Homogeneous erythropoietin characterized by a molecular weight of about 34,000 daltons on SDS PAGE, movement as a single peak on reverse phase high performance liquid chromatography and a specific activity of at least about 160,000 IU per absorbance unit at 280 nanometers.

6. A pharmaceutical composition for the treatment of anemia comprising a therapeutically effective amount of the homogeneous erythropoietin of claim 4 in a pharmaceutically acceptable vehicle.

Dr. Hewick assigned the patent to GI.

The other patent in this litigation is U.S. Patent 4,703,008, entitled "DNA Sequences Encoding Erythropoietin" (the '008 patent), issued on October 27, 1987, to Dr. Fu-Kuen Lin, an employee of Amgen. The claims of the '008 patent cover purified and isolated DNA sequences encoding erythropoietin and host cells transformed or transfected with a DNA sequence. The relevant claims are as follows:

2. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding human erythropoietin.

4. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to claim 1, 2 or 3 in a manner allowing the host cell to express erythropoietin.

6. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to claim 5.

7. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake.

8. A cDNA sequence according to claim 7.

23. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to claim 7, 8, or 11 in a manner allowing the host cell to express said polypeptide.

24. A transformed or transfected host cell according to claim 23 which host cell is capable of glycosylating said polypeptide.

25. A transformed or transfected mammalian host cell according to claim 24.

26. A transformed or transfected COS cell according to claim 25.

27. A transformed or transfected CHO cell according to claim 25.

29. A procaryotic host cell stably transformed or transfected with a DNA vector according to claim 28.

PROCEDURAL HISTORY

On October 27, 1987, the same day that the '008 patent was issued, Amgen filed suit against Chugai and GI. It alleged that GI infringed the '008 patent by the production of recombinant EPO (rEPO) and by use of transformed mammalian host cells containing vectors with DNA coding for the production of human EPO, and that Chugai, as a result of a collaborative relationship with GI, had induced and/or contributed to the direct infringement of the '008 patent by GI. Amgen further sought a declaration that GI's '195 patent is invalid under 35 U.S.C. §§102, 103,

and 112, or, in the alternative, that Amgen does not infringe the claims of the '195 patent, and a declaration that GI and Chugai's future activities in the production and sale of rEPO will infringe the '008 patent. 2

GI and Chugai answered and counterclaimed, asserting several affirmative defenses, including invalidity under 35 U.S.C. §§101, 102, 103, and 112; non-infringement; failure to make deposits at a public depository of biological materials allegedly necessary for enabling the best mode of practicing the invention; and unenforceability of the

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patent because of Amgen's alleged inequitable conduct before the PTO. GI also counterclaimed, alleging that Amgen infringed the '195 patent, asserting unfair competition, and seeking a declaratory judgment that the '008 patent was invalid and not infringed.

GI and Chugai then filed a joint motion for a partial summary judgment that Amgen infringed the claims of the '195 patent. Chugai also filed its own motion for summary judgment. On February 24, 1988, the district court granted GI's and Chugai's motion for partial summary judgment and, on January 31, 1989, the court granted Chugai's motion for partial summary judgment only to the extent of ruling that the '008 patent does not contain a process claim, an issue that is not now before us.

In response to Amgen's motion for a preliminary injunction, the district court, on February 7, 1989, issued an order finding that "Amgen had shown a reasonable likelihood of success on the merits of the validity of its patent; that it would suffer irreparable injury due to the needs of an incipient market and the attendant burdens on a new company; ..." and that, as to the public interest, "recombinant EPO is an extraordinarily valuable medicine that promises marked relief from renal failure." Because of this public interest finding, the court determined that it would not enter an order to delay or prevent production or shipping of EPO, but would require the defendant GI to place with the court all profits from the sale of EPO.

In order to expedite trial, the parties consented to trial before a magistrate. The judge entered judgment upon findings of fact and conclusions of law set forth by the magistrate. With respect to Amgen's '008 patent, the court held that claims 2, 4, and 6 are valid, enforceable and have been infringed by GI; that infringement was not willful; that claims 7, 8, 23-27, and 29 are invalid for lack of enablement under 35 U.S.C. §112 but, if valid, were infringed by GI; that the '008 patent does not contain a process claim; and that Chugai has not infringed, contributorily infringed, or induced infringement of any claim of the '008 patent. The court also dismissed Amgen's complaint against Chugai.

With respect to GI's '195 patent, the court concluded that claims 1 and 3 are valid, enforceable, and have been infringed by Amgen; that Amgen has not infringed claims 2 and 5; that Amgen's infringement was not willful; and that claims 4 and 6 are invalid for indefiniteness under 35 U.S.C. §112, but, if valid, were infringed by Amgen. The court also concluded that Amgen did not misuse the '008 patent and that this was not an "exceptional" case under 35 U.S.C. §285.

DISCUSSION

I. AMGEN'S '008 PATENT (Lin)

A. *Alleged prior invention under 35 U.S.C. §102(g)*

The first issue we review is whether the district court erred in finding that the claims directed to a purified and isolated DNA sequence encoding human EPO were not invalidated by the work of GI's Dr. Fritsch. Section 102(g) provides in relevant part that:

A person is entitled to a patent unless-(g) before the applicant's invention thereof the invention was made ... by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the

reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other.

Defendants assert error in the district court's legal conclusion that in this case Lin's conception occurred simultaneously with reduction to practice. See e.g., *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1376, 231 USPQ 81, 87 (Fed. Cir. 1988), *cert. denied*, 480 U.S. 947 (1987). They claim that Fritsch was first to conceive a probing strategy of using two sets of fully-degenerate cDNA probes of two different regions of the EPO gene to screen a gDNA library, which was the strategy which the district court found eventually resulted in the successful identification and isolation of the EPO gene. Defendants further claim that Fritsch conceived this strategy in 1981, was diligent until he reduced the invention to practice in May of 1984, and thus should be held to be a 102(g) prior inventor over Lin, who reduced the invention to practice in September of 1983.

Conception is the "formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice." *Hybritech*, 802 F.2d at 1376, 231 USPQ at 87 (citing 1 *Robinson on Patents* 532 (1890)); *Coleman v. Dines*, 754 F.2d 353, 359, 224 USPQ 857, 862 (Fed. Cir. 1985) (citing *Gunter v. Stream*, 573 F.2d 77, 80, 197 USPQ 482, 484 (CCPA 1978)). Conception requires both the idea of the inven

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tion's structure and possession of an operative method of making it. *Oka v. Youssefeyeh*, 849 F.2d 581, 583, 7 USPQ2d 1169, 1171 (Fed. Cir. 1988).

In some instances, an inventor is unable to establish a conception until he has reduced the invention to practice through a successful experiment. This situation results in a simultaneous conception and reduction to practice. See 3 D. Chisum, *Patents* §10.04[5] (1990). We agree with the district court that that is what occurred in this case. The invention recited in claim 2 is a "purified and isolated DNA sequence" encoding human EPO. The structure of this DNA sequence was unknown until 1983, when the gene was cloned by Lin; Fritsch was unaware of it until 1984. As Dr. Sadler, an expert for GI, testified in his deposition: "You have to clone it first to get the sequence." In order to design a set of degenerate probes, one of which will hybridize with a particular gene, the amino acid sequence, or a portion thereof, of the protein of interest must be known. Prior to 1983, the amino acid sequence for EPO was uncertain, and in some positions the sequence envisioned was incorrect. Thus, until Fritsch had a complete mental conception of a purified and isolated DNA sequence encoding EPO and a method for its preparation, in which the precise identity of the sequence is envisioned, or in terms of other characteristics sufficient to distinguish it from other genes, all he had was an objective to make an invention which he could not then adequately describe or define.

[1] A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. See *Oka*, 849 F.2d at 583, 7 USPQ2d at 1171. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, e.g., encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, i.e., until after the gene has been isolated.

Fritsch had a goal of obtaining the isolated EPO gene, whatever its identity, and even had an idea of a possible method of obtaining it, but he did not conceive a purified and isolated DNA sequence encoding EPO and a viable method for obtaining it until after Lin. It is important to recognize that neither Fritsch nor Lin invented EPO or the EPO gene. The subject matter of claim 2 was the novel *purified and isolated* sequence which codes for EPO, and

neither Fritsch nor Lin knew the structure or physical characteristics of it and had a viable method of obtaining that subject matter until it was actually obtained and characterized.

[2] Defendants further argue that because the trial court found that the probing and screening method employed by Lin is what distinguished the invention of the '008 patent over the prior art, Fritsch's strategy in 1981 had priority over Lin's use of that strategy. We disagree. The trial court found that Fritsch's alleged conception in 1981 of an approach that might result in cloning the gene was mere speculation. Conception of a generalized approach for screening a DNA library that might be used to identify and clone the EPO gene of then unknown constitution is not conception of a "purified and isolated DNA sequence" encoding human EPO. It is not "a definite and permanent idea of the complete and operative invention." Fritsch's conception of a process had to be sufficiently specific that one skilled in the relevant art would succeed in cloning the EPO gene. *See Coleman*, 754 F.2d at 359, 224 USPQ at 862. Clearly, he did not have that conception because he did not know the structure of EPO or the EPO gene. The record indicates that several companies, as well as Amgen and GI, were unsuccessful using Fritsch's approach. As the trial court correctly summarized:

Given the utter lack of experience in probing genomic libraries with fully degenerate probes and the crudeness of the techniques available in 1981, it would have been mere speculation or at most a probable deduction from facts then known by Dr. Fritsch that his generalized approach would result in cloning the EPO gene.

13 USPQ2d at 1760. As expert testimony from both sides indicated, success in cloning the EPO gene was not assured until the gene was in fact isolated and its sequence known. Based on the uncertainties of the method and lack of information concerning the amino acid sequence of the EPO protein, the trial court was correct in concluding that neither party had an adequate conception of the DNA sequence until reduction to practice

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had been achieved; Lin was first to accomplish that goal.

Defendants also argue that the court failed to consider that 1983, just prior to Lin's conception, was the relevant time for determining the completeness of Fritsch's conception, not 1981. However, the record shows that the court did consider what occurred in 1983. Moreover, Fritsch had no more of a conception in 1983 than he did in 1981, because he did not then know the sequence of the gene encoding EPO.

B. Alleged obviousness of the inventions of claims 2, 4, and 6

Claim 2, as noted above, recites a purified and isolated DNA sequence, and claims 4 and 6 are directed to host cells transformed with such a DNA sequence. The district court determined that claims 2, 4, and 6 are not invalid under 35 U.S.C. §103, concluding that the unique probing and screening method employed by Lin in isolating the EPO gene and the extensive effort required to employ that method made the invention nonobvious over the prior art. 3

Obviousness under Section 103 is a question of law. *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561, 1568, 1 USPQ2d 1593, 1597 (Fed. Cir.), *cert. denied*, 481 U.S. 1052 (1987). The district court stated that one must inquire whether the prior art would have suggested to one of ordinary skill in the art that Lin's probing and screening method should be carried out and would have a reasonable expectation of success, viewed in light of the prior art. *See In re Dow Chemical Co.*, 837 F.2d 469 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). "Both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure." *Id.*

[3] The district court specifically found that, as of 1983, none of the prior art references "suggest[s] that the probing strategy of using two fully-redundant [sic] sets of probes, of relatively high degeneracy [sic], to screen a human genomic library would be likely to succeed in pulling out the gene of interest." 4 13 USPQ2d at 1768.

While it found that defendants had shown that these procedures were "obvious to try," the references did not show that there was a reasonable expectation of success. *See In re O'Farrell*, 853 F.2d 894, 903-04, 7 USPQ2d 1673, 1680-81 (Fed. Cir. 1988).

Defendants challenge the district court's determination, arguing that, as of September 1983, one of ordinary skill in the art would have had a reasonable expectation of success in screening a gDNA library by Lin's method in order to obtain EPO. We agree with the district court's conclusion, which was supported by convincing testimony. One

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witness, Dr. Davies of Biogen, another biotechnology company that had worked on EPO, stated that he could not say whether Biogen scientists would have succeeded in isolating the EPO gene if Biogen had the EPO fragments that were available to Lin in 1983. Dr. Wall, a professor at UCLA, testified that it would have been "difficult" to find the gene in 1983, and that there would have been no more than a fifty percent chance of success. He said, "you couldn't be certain where in the genomic DNA your probe might fall." The court found that no one had successfully screened a genomic library using fully-degenerate probes of such high redundancy as the probes used by Lin. In the face of this and other evidence on both sides of the issue, it concluded that defendants had not shown by clear and convincing evidence that the procedures used by Lin would have been obvious in September 1983. We are not persuaded that the court erred in its decision.

Defendants assert that whether or not it would have been obvious to isolate the human EPO gene from a gDNA library with fully-degenerate probes is immaterial because it was obvious to use the already known monkey EPO gene as a probe. Defendants point out that, in the early 1980s, Biogen did significant work with an EPO cDNA obtained from a baboon, and that they used it as a probe to hybridize with the corresponding gene in a human gDNA library. However, this technique did not succeed until after Lin isolated the EPO gene with his fully-degenerate set of probes.

To support its obviousness assertion, defendants rely upon the testimony of their expert, Dr. Flavell, who testified that the overall homology of baboon DNA and human DNA was "roughly 90 percent". While this testimony indicates that it might have been feasible, perhaps obvious to try, to successfully probe a human gDNA library with a monkey cDNA probe, it does not indicate that the gene could have been identified and isolated with a reasonable likelihood of success. Neither the DNA nucleotide sequence of the human EPO gene nor its exact degree of homology with the monkey EPO gene was known at the time.

Indeed, the district court found that Lin was unsuccessful at probing a human gDNA library with monkey cDNA until after he had isolated the EPO gene by using the fully-degenerate probes. Based on the evidence in the record, the district court found there was no reasonable expectation of success in obtaining the EPO gene by the method that Lin eventually used. While the idea of using the monkey gene to probe for a homologous human gene may have been obvious to try, the realization of that idea would not have been obvious. There were many pitfalls. Hindsight is not a justifiable basis on which to find that ultimate achievement of a long sought and difficult scientific goal was obvious. The district court thoroughly examined the evidence and the testimony. We see no error in its result. Moreover, if the DNA sequence was not obvious, host cells containing such sequence, as claimed in claims 4 and 6, could not have been obvious. We conclude that the district court did not err in holding that the claims of the patent are not invalid under Section 103.

C. Best Mode

Defendants argue that the district court erred in failing to hold the '008 patent invalid under 35 U.S.C. §112, asserting that Lin failed to disclose the best mammalian host cells known to him as of November 30, 1984, the date he filed his fourth patent application.

The district court found that the "best mode" of practicing the claimed invention was by use of a specific genetically-heterogeneous strain of Chinese hamster ovary (CHO) cells, which produced EPO at a rate greater than that of other cells. It further found that this strain was disclosed in Example 10 and that Lin knew of no better mode. GI argues that Lin's best mode was not adequately disclosed in Example 10 because one skilled in the art could not duplicate Lin's best mode without his having first deposited a sample of the specific cells in a public

depository. The issue before us therefore is whether the district court erred in concluding that Example 10 of the '008 patent satisfied the best mode requirement as to the invention of the challenged claims 5 and that a deposit of the preferred CHO cells was not necessary.

[4] A determination whether the best mode requirement is satisfied is a question of fact, *DeGeorge v. Bernier*, 768 F.2d 1318, 1324, 226 USPQ 758, 763 (Fed. Cir. 1985); we

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therefore review the district court's finding under a clearly erroneous standard.

35 U.S.C. §112 provides in relevant part: The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, *and shall set forth the best mode contemplated by the inventor of carrying out his invention.*

(Emphasis added).

This court has recently discussed the best mode requirement, pointing out that its analysis has two components. *Chemcast Corp. v. Arco Indus. Corp.*, 913 F.2d 923, 927, 16 USPQ2d 1033, 1036 (Fed. Cir. 1990). The first is a subjective one, asking whether, at the time the inventor filed his patent application, he contemplated a best mode of practicing his invention. If he did, the second inquiry is whether his disclosure is adequate to enable one skilled in the art to practice the best mode or, in other words, whether the best mode has been concealed from the public. The best mode requirement thus is intended to ensure that a patent applicant plays "fair and square" with the patent system. It is a requirement that the *quid pro quo* of the patent grant be satisfied. One must not receive the right to exclude others unless at the time of filing he has provided an adequate disclosure of the best mode known to him of carrying out his invention. Our case law has interpreted the best mode requirement to mean that there must be no concealment of a mode known by the inventor to be better than that which is disclosed. *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 1384-85, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987). Section 282 imposes on those attempting to prove invalidity the burden of proof. We agree that the district court did not err in finding that defendants have not met their burden of proving a best mode violation. As noted above, the district court found that the best mode of making the CHO cells was set forth in Example 10. As the district court stated, while it was not clear which of two possible strains Lin considered to be the best, the cell strain subjected to 1000 nanomolar MTX (methotrexate) or that subjected to 100 nanomolar MTX, the best mode was disclosed because both were disclosed. 6 Defendants argue that this disclosure is not enough, that a deposit of the cells was required.

Defendants contend that "[i]n the field of living materials such as microorganisms and cell cultures," we should require a biological deposit so that the public has access to exactly the best mode contemplated by the inventor. This presents us with a question of first impression concerning the best mode requirement for patents involving novel genetically-engineered biological subject matter.

For many years, it has been customary for patent applicants to place microorganism samples in a public depository when such a sample is necessary to carry out a claimed invention. This practice arose out of the development of antibiotics, when microorganisms obtained from soil samples uniquely synthesized antibiotics which could not be readily prepared chemically or otherwise. *In re Argoudelis*, 434 F.2d 1390, 168 USPQ 99 (CCPA 1970). Such a deposit has been considered adequate to satisfy the *enablement* requirement of 35 U.S.C. §112, when a written description alone would not place the invention in the hands of the public and physical possession of a unique biological material is required. *See, e.g., In re Wands*, 858 F.2d 731, 735-36, 8 USPQ2d 1400, 1403 (Fed. Cir. 1988) ("Where an invention depends on the use of living materials ... it may be impossible to enable the public to make the invention (*i.e.*, to obtain these living materials) solely by means of written disclosure."); *In re Lundak*, 773 F.2d 1216, 1220, 227 USPQ 90, 93 (Fed. Cir. 1985) ("When an invention relates to a new biological material, the material may not be reproducible even when detailed procedures and a complete taxonomic description are

included in the specification."); *see generally* Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement*, 67 J. Pat. & Trademark Off. Soc'y 569, 607 (1985) ("The deposit requirement is a non-statutory mechanism for ensuring compliance with the 'enabling' provision under 35 U.S.C. § 112."). The district court found that the claims at issue require the use of biological materials

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that were capable of being prepared in the laboratory from readily available biological cells, using the description in Example 10. The court also found that there were no starting materials that were not publicly available, that were not described, or that required undue experimentation for their preparation in order to carry out the best mode. The court noted that Lin testified that the isolation of the preferred strain was a "routine limited dilution cloning procedure[]" well known in the art. Dr. Simonsen, GI's own expert, testified that the disclosed procedures were "standard" and that:

with the vectors and the sequences shown in Example 10, I have no doubt that someone eventually could reproduce-well, could generate cell lines [sic, strains] making some level of EPO, and they could be better, they could be worse in terms of EPO production.

The district court relied on this testimony, and, upon review, we agree with its determination. The testimony accurately reflects that the invention, as it relates to the *best mode* host cells, could be practiced by one skilled in the art following Example 10. Thus, the best mode was disclosed and it was adequately enabled.

[5] These materials are therefore not analogous to the biological cells obtained from unique soil samples. When a biological sample required for the practice of an invention is obtained from nature, the invention may be incapable of being practiced without access to that organism. Hence the deposit is required in that case. On the other hand, when, as is the case here, the organism is created by insertion of genetic material into a cell obtained from generally available sources, then all that is required is a description of the best mode and an adequate description of the means of carrying out the invention, not deposit of the cells. If the cells can be prepared without undue experimentation from known materials, based on the description in the patent specification, a deposit is not required. *See Feldman v. Aunstrup*, 517 F.2d 1351, 1354, 186 USPQ 108, 111 (CCPA 1975), ("No problem exists when the microorganisms used are known and readily available to the public."), *cert. denied*, 424 U.S. 912 [188 USPQ 720] (1976). Since the court found that that is the case here, we therefore hold that there is no failure to comply with the best mode requirement for lack of a deposit of the CHO cells, when the *best mode* of preparing the cells has been disclosed and the best mode cells have been enabled, *i.e.*, they can be prepared by one skilled in the art from known materials using the description in the specification.

Defendants also contend that the examiner's rejection of the application that matured into the '008 patent for failure to make a publicly accessible biological deposit supports its argument. U.S. Patent Application Serial No. 675,298, Prosecution History at 179 (First Rejection July 3, 1986). However, that rejection was withdrawn after an oral interview and a written argument that the invention did not require a deposit. *Id.* at 208.

We also note that the PTO has recently prescribed guidelines concerning the deposit of biological materials. *See* 37 C.F.R. §1.802(b) (1990) (biological material need not be deposited "if it is known and readily available to the public or can be made or isolated without undue experimentation"). The PTO, in response to a question as to whether the deposit requirement is applicable to the best mode requirement, as distinct from enablement, said: The best mode requirement is a safeguard against the possible selfish desire on the part of some people to obtain patent protection without making a full disclosure. The requirement does not permit an inventor to disclose only what is known to be the second-best embodiment, retaining the best The fundamental issue that should be addressed is whether there was evidence to show the quality of an applicant's best mode disclosure is so poor as to effectively result in concealment. *In re Sherwood*, 615 F.2d 809, 204 USPQ 537 (CCPA 1980). If a deposit is the only way to comply with the best mode requirement then the deposit must be made.

52 Fed.Reg. 34080, 34086 (Sept. 8, 1987). 7

We see no inconsistency between the district court's decision, which we affirm here, and these guidelines. [6] Defendants also assert that the record shows that scientists were unable to duplicate Lin's genetically-heterogeneous best mode cell strain. However, we have long held that the issue is whether the disclosure is "adequate," not that an exact duplication is necessary. Indeed, the district court stated that he testimony is clear that no scientist could ever duplicate exactly the best mode used by Amgen, but that those of ordinary skill in the art could produce mammalian

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host cell strains or lines with similar levels of production identified in Example 10.

13 USPQ2d at 1774. What is required is an adequate disclosure of the best mode, not a guarantee that every aspect of the specification be precisely and universally reproducible. *See In re Gay*, 309 F.2d 769, 773, 135 USPQ 311, 316 (CCPA 1962).

Defendants finally argue that Lin's failure to deposit the transfected cells notwithstanding the fact that he was willing to deposit essentially worthless cell material was evidence of deliberate concealment. We have already stated that deposit of the host cells containing the rEPO gene was not necessary to satisfy the best mode requirement of Section 112. The best mode was disclosed and a deposit was not necessary to carry it out. Therefore, the fact that some cells were deposited, but not others, is irrelevant.

D. Enablement of claims 7, 8, 23-27, and 29

Amgen argues that the district court's holding that GI "provided clear and convincing evidence that the patent specification is insufficient to enable one of ordinary skill in the art to make and use the invention claimed in claim 7 of the '008 patent without undue experimentation" constituted legal error. 13 USPQ2d at 1776. Amgen specifically argues that the district court erred because it "did not properly address the factors which this court has held must be considered in determining lack of enablement based on assertion of undue experimentation," citing this court's decision in *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

Claim 7 is a generic claim, covering all possible DNA sequences that will encode any polypeptide having an amino acid sequence "sufficiently duplicative" of EPO to possess the property of increasing production of red blood cells. As claims 8, 23-27, and 29, dependent on claim 7, are not separately argued, and are of similar scope, they stand or fall with claim 7. *See In re Dillon*, 919 F.2d 688, 692, 16 USPQ2d 1897, 1900 (Fed. Cir. 1990) (in banc).

[7] Whether a claimed invention is enabled under 35 U.S.C. §112 is a question of law, which we review *de novo*. *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 1268, 229 USPQ 805, 811 (Fed. Cir. 1986), *cert. denied*, 479 U.S. 1030 (1987). "To be enabling under §112, a patent must contain a description that enables one skilled in the art to make and use the claimed invention." *Atlas Powder Co. v. E.I. duPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984).

That some experimentation is necessary does not constitute a lack of enablement; the amount of experimentation, however, must not be unduly extensive. *Id.* The essential question here is whether the scope of enablement of claim 7 is as broad as the scope of the claim. *See generally In re Fisher*, 427 F.2d 833, 166 USPQ 18 (CCPA 1970); 2 D. Chisum, *Patents* §7.03[7][b] (1990).

The specification of the '008 patent provides that:

one may readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for mature EPO in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions).

DNA sequences provided by the present invention are thus seen to comprehend all DNA sequences suitable for use in securing expression in a procaryotic or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin, and selected from among: (a) the DNA sequences set out in FIGS. 5 and 6; (b) DNA sequences which hybridize to the DNA

sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

The district court found that over 3,600 different EPO analogs can be made by substituting at only a single amino acid position, and over a million different analogs can be made by substituting three amino acids. The patent indicates that it embraces means for preparation of "numerous" polypeptide analogs of EPO. Thus, the number of claimed DNA encoding sequences that can produce an EPO-like product is potentially enormous.

In a deposition, Dr. Elliott, who was head of Amgen's EPO analog program, testified that he did not know whether the fifty to eighty EPO analogs Amgen had made "had the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake." Based on this evidence, the trial court concluded that "defendants had provided clear and convincing evidence that the patent specification is insufficient to enable one of ordinary skill in the art to make and use the invention claimed in claim 7 of the '008 patent without undue experimentation." 13 USPQ at 1776. In making this determina-

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tion, the court relied in particular on the lack of predictability in the art, as demonstrated by the testimony of both Dr. Goldwasser, another scientist who worked on procedures for purifying urinary EPO (uEPO), and Dr. Elliott. After five years of experimentation, the court noted, "Amgen is still unable to specify which analogs have the biological properties set forth in claim 7." *Id.*

We believe the trial court arrived at the correct decision, although for the wrong reason. By focusing on the biological properties of the EPO analogs, it failed to consider the enablement of the DNA sequence analogs, which are the subject of claim 7. Moreover, it is not necessary that a patent applicant test all the embodiments of his invention, *In re Angstadt*, 537 F.2d 498, 502, 190 USPQ 214, 218 (CCPA 1976); what is necessary is that he provide a disclosure sufficient to enable one skilled in the art to carry out the invention commensurate with the scope of his claims. For DNA sequences, that means disclosing how to make and use enough sequences to justify grant of the claims sought. Amgen has not done that here. In addition, it is not necessary that a court review all the *Wands* factors to find a disclosure enabling. They are illustrative, not mandatory. What is relevant depends on the facts, and the facts here are that Amgen has not enabled preparation of DNA sequences sufficient to support its all-encompassing claims.

[8] It is well established that a patent applicant is entitled to claim his invention generically, when he describes it sufficiently to meet the requirements of Section 112. *See Utter v. Hiraga*, 845 F.2d 993, 998, 6 USPQ2d 1709, 1714 (Fed. Cir. 1988) ("A specification may, within the meaning of 35 U.S.C. §112¶1, contain a written description of a broadly claimed invention without describing all species that claim encompasses."); *In re Robins*, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (CCPA 1970) ("[R]epresentative samples are not required by the statute and are not an end in themselves."). Here, however, despite extensive statements in the specification concerning all the analogs of the EPO gene that can be made, there is little enabling disclosure of particular analogs and how to make them. Details for preparing only a few EPO analog genes are disclosed. Amgen argues that this is sufficient to support its claims; we disagree. This "disclosure" might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for Amgen's desire to claim all EPO gene analogs. There may be many other genetic sequences that code for EPO-Type products. Amgen has told how to make and use only a few of them and is therefore not entitled to claim all of them.

In affirming the district court's invalidation of claims 7, 8, 23-27, and 29 under Section 112, we do not intend to imply that generic claims to genetic sequences cannot be valid where they are of a scope appropriate to the invention disclosed by an applicant. That is not the case here, where Amgen has claimed every possible analog of a gene containing about 4,000 nucleotides, with a disclosure only of how to make EPO and a very few analogs. The district court properly relied upon *Fisher* 8 in making its decision. In that case, an applicant was attempting to claim an adrenocorticotrophic hormone preparation containing a polypeptide having at least twenty-four amino

acids of a specified sequence. Only a thirty-nine amino acid product was disclosed. The court found that applicant could not obtain claims that are insufficiently supported and hence not in compliance with the first paragraph of 35 U.S.C. §112. It stated:

Appellant's parent application, therefore, discloses no products, inherently or expressly, containing other than 39 amino acids, yet the claim includes all polypeptides, of the recited potency and purity, having at least 24 amino acids in the chain in the recited sequence. The parent specification does not enable one skilled in the art to make or obtain ACTHs with other than 39 amino acids in the chain, and there has been no showing that one of ordinary skill would have known how to make or obtain such other ACTHs without undue experimentation. As for appellant's conclusion that the 25th to 39th acids in the chain are unnecessary, it is one thing to make such a statement when persons skilled in the art are able to make or obtain ACTH having other than 39 amino acids; it is quite another thing when they are not able to do so. In the latter situation, the statement is in no way "enabling" and hence lends no further support for the broad claim. We conclude that appellant's parent applica

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tion is insufficient to support a claim as broad as claim 4.

requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art.

Fisher, 427 F.2d at 836, 839, 166 USPQ at 21-2224.

Considering the structural complexity of the EPO gene, the manifold possibilities for change in its structure, with attendant uncertainty as to what utility will be possessed by these analogs, we consider that more is needed concerning identifying the various analogs that are within the scope of the claim, methods for making them, and structural requirements for producing compounds with EPO-like activity. It is not sufficient, having made the gene and a handful of analogs whose activity has not been clearly ascertained, to claim all possible genetic sequences that have EPO-like activity. Under the circumstances, we find no error in the court's conclusion that the generic DNA sequence claims are invalid under Section 112.

E. Inequitable Conduct

Defendants argue that the '008 patent claims are unenforceable as a result of an asserted misrepresentation of the number of probes Lin used for the monkey gene cloning described in Example 3 of his patent. Relying on the district court's finding that Lin had said that a "full set" mixture of 128 "EpV" probes was used for monkey cDNA screening, whereas only a 16-member "subset" of the EpV mixture was actually used, defendants argue that the court ought to have found that the representations were material.

[9] The essential elements of proof of inequitable conduct include intent to deceive and materiality. After finding threshold levels of materiality and intent, the trial court must balance the two and determine, in its discretion, whether inequitable conduct has occurred. *J.P. Stevens & Co. v. Lex Tex Ltd., Inc.*, 747 F.2d 1553, 1560, 223 USPQ 1089, 1092 (Fed. Cir. 1984), *cert. denied*, 474 U.S. 822 (1985). While we review an ultimate conclusion of inequitable conduct under an abuse of discretion standard, *Kingsdown Medical Consultants, Ltd. v. Hollister Inc.*, 863 F.2d 867, 876, 9 USPQ2d 1384, 1392 (Fed. Cir. 1988) (in banc), *cert. denied*, 490 U.S. 1067 (1989), the underlying factual threshold findings are reviewed under a clearly erroneous standard.

Lin set out to clone the EPO gene by more than one method, including using degenerate human probes and monkey probes. It is not disputed that he did isolate the human EPO gene from a genomic library using two different 128-member pools of probes made from fragments of the human EPO protein. Thereafter, he also attempted to use the human sequence probes to find the monkey EPO cDNA to be used later as a probe to hybridize with the human EPO gene. Example 3 of the '008 patent describes this work, indicating that the screening yielded seven positive clones. It also reports that a subset of the human EpV mixture was used for DNA sequencing work. When Lin published his monkey cDNA cloning work in a scientific journal, he also reported the

use of 128 EpV probes to screen the monkey library. Lin screened the monkey library with the full mixture of 128 EpV probes and with one of eight subsets of probes which made up the full EpV mixture. In response to a question whether a subset of EpV probes was used in the first screening of the monkey cDNA library, Lin testified: I don't know which we used, the subset first or used the full set first. I cannot recall exactly. It looks like the subset was first defining the number, yes.

This answer constituted the sole basis for the court's finding that, "[a]t trial, Lin admitted he only used a subset of the EpV 128 probes in screening the cDNA library." 13 USPQ2d at 1778.

We consider that the district court's finding of an "admission" of misrepresentation in Lin's testimony and its conclusion that GI "presented clear and convincing evidence of a misrepresentation" was clearly erroneous. That Lin did not recall whether he first screened the monkey cDNA library with a full set of probes or a subset of probes, and his answer that "it looks like" he used the subset, are certainly not clear admissions that he only used a subset. However, the district court was correct in concluding that, even if there had been an erroneous statement, it was not material because Lin succeeded in cloning the EPO gene first with his use of the fully-degenerate probes. Thus, his testimony does not provide clear and convincing evidence that he misrepresented to the PTO the number of probes used. He did use 128-member probes as well as a subset. Moreover, this evidence does not create an inference of an intent to mislead. The court

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properly concluded that there was no inequitable conduct in prosecuting the '008 patent.

II. GI's '195 PATENT (Hewick)

A. Enablement of claims 1 and 3

Amgen challenges the district court's determination that "the '195 patent enables a person of ordinary skill in the art to obtain homogeneous EPO [including rEPO and uEPO] from natural sources" having a mean *in vivo* specific activity of at least 160,000. 10 13 USPQ2d at 1794. Claims 1 and 3 contain the limitation that EPO have a specific activity of at least 160,000 IU/AU. The district court found, based upon expert testimony from both sides, that to those skilled in the art, in the absence of an express statement in the patent, the claims would be construed to refer to *in vivo* rather than *in vitro* specific activity. To support its challenge, Amgen asserts that the district court's determination is contradicted by GI's own bioassay data and by the district court's finding that "the '195 patent fails to enable the purification of rEPO." Amgen also asserts that the district court erred in relying solely on an *in vitro* measure of specific activity, having initially construed the '195 claims as requiring an *in vivo* measure to avoid invalidity for indefiniteness.

35 U.S.C. §112 requires that an invention be described "in such full, clear, concise, and exact terms as to enable any person skilled in the art ... to make and use the same." We review a determination of enablement as a question of law. *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 1268, 229 USPQ 805, 811 (Fed. Cir. 1986), *cert. denied*, 479 U.S. 1030 (1987).

We do not consider the court's finding that the assay measurement was an *in vivo* one to be erroneous in view of the testimony it heard. That being the case, the question is whether the court erred in concluding that the claims requiring 160,000 IU/AU by an *in vivo* measurement were enabled. We conclude that it did err.

Defendants have produced no evidence that it ever prepared EPO with a specific activity of at least 160,000 IU/AU *in vivo* using the disclosed methods. In its report to the FDA, GI stated that it had purified uEPO material "to homogeneity" by subjecting partially purified uEPO material to reverse phase high performance liquid chromatography (RP-HPLC), the technique taught by Hewick in the '195 patent. The district court found that GI reported to the FDA that the specific activity of uEPO, based on *in vivo* bioassays, was only 109,000 IU/AU. 11 GI originally arrived at the figure of 160,000 IU/AU by calculation, before it had the capacity to derive

quantitative information from bioassays. Hewick subjected the EPO to RP-HPLC, the EPO having an actual value of 83,000 IU/AU. After weighing the chromatograph, he found that "at least fifty percent" of the area under the chromatograph curve was attributable to something other than EPO. He then doubled the 83,000, and arrived at a theoretical specific activity of "at least about 160,000 IU/AU." That procedure, while possibly valid as a means for estimating the specific activity of a pure sample, does not establish that GI had a workable method for actually obtaining the pure material that it claimed.

Moreover, the work of others shows that Hewick did not enable the preparation of uEPO having an *in vivo* specific activity of at least 160,000, as the claims required. Dr. Kawakita, a scientist at Kumamoto University in Japan, reported an *in vivo* specific activity of 101,000 IU/AU when using RP-HPLC according to Hewick's method. This is similar to the 109,000 value reported to the FDA by GI. Kawakita did report a value of 188,000, but did not follow the teachings in the '195 patent. Defendants also rely on the testimony of Fritsch that "I've also seen further data in Chugai's PLA indicating additional urinary EPO preparation that had activities of 190,000, I believe, units per absorbance unit." However, the document to which Fritsch referred was not offered into evidence by GI after Amgen objected to its introduction and is not before us.

Defendants argue that Dr. Kung's uEPO test result of 173,640 IU/AU in an *in vitro* test supports the enablement of its claims. Amgen argues that an *in vivo* test result would only have been 65 percent of the *in vitro* result and thus would not have met the 160,000 IU/AU limitation of the claims. The district court relied on Kung, despite the demonstrated disparity between the results of *in vitro* and *in vivo* testing.

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[10] It is not absolutely clear to us that, for uEPO, the *in vivo* specific activity is 65 percent of the *in vitro* specific activity. Nonetheless, Kung's measurement, being *in vitro*, does not demonstrate enablement of the claimed invention, and that fact means that the court erred in finding enablement. Added to this fact is the difference that exists between the *in vivo* results for rEPO and uEPO 12, and the other lack of support for the 160,000 limitation. Under these circumstances, we hold that the district court erred in accepting the *in vitro* data as support for claims containing what has been found to be an *in vivo* limitation.

In addition to the question of enablement regarding uEPO, the district court found that the only purification attempt on rEPO in the manner set out in the '195 patent failed to provide homogeneous EPO. The patent itself, in Example 2, discloses GI's purification efforts on rEPO and indicates that GI did not obtain purified rEPO. As the district court found, "[t]he patent does not contain any procedures ... for purifying rEPO to the point that RP-HPLC will be successful." 13 USPQ2d at 1758. Thus, the patent fails to enable purification of either rEPO or uEPO. 13 *See In re Rainer*, 377 F.2d 1006, 1012, 153 USPQ 802, 807 (CCPA 1967) ("specification is evidence of its own inadequacy").

The burden of showing non-enablement is Amgen's, not GI's, but in the case of a challenged patent, when substantial discovery has occurred, and there is no credible evidence that the claimed purified material can be made by those skilled in the art by the disclosed process, and all evidence from both the inventor and his assignee and from third parties is to the contrary, we conclude that Amgen has met its burden to show that the claims have not been adequately enabled. We do not hold that one must always prove that a disclosed process operates effectively to produce a claimed product. But, under these circumstances, we conclude that the court erred in holding that claims 1 and 3 were properly enabled.

B. Indefiniteness of claims 4 and 6

The district court held claims 4 and 6 of the '195 patent invalid because their specific activity limitation of "at least about 160,000" was indefinite. Defendants challenge this holding, asserting that there is no evidence that claims 4 and 6 do not comply with the requirements of 35 U.S.C. §112.

The statute requires that "[t]he specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention." A decision as to whether a claim is invalid under this provision requires a determination whether those skilled in the art would understand what is claimed. See *Shatterproof Glass Corp. v. Libbey-Owens Ford Co.*, 758 F.2d 613, 624, 225 USPQ 634, 641 (Fed. Cir. 1985) (Claims must "reasonably apprise those skilled in the art" as to their scope and be "as precise as the subject matter permits."). The district court found that "bioassays provide an imprecise form of measurement with a range of error" and that use of the term "about" 160,000 IU/AU, coupled with the range of error already inherent in the specific activity limitation, served neither to distinguish the invention over the close prior art (which described preparations of 120,000 IU/AU), nor to permit one to know what specific activity values below 160,000, if any, might constitute infringement. 13 USPQ2d at 1787. It found evidence of ambiguity in the fact that Chugai, GI's partner, itself questioned whether the specific activity value of 138,000 IU/AU for its own rEPO was within the claim coverage.

In prosecuting the '195 patent, GI disclosed to the examiner a publication by Miyake et al., which discloses a uEPO product having an *in vivo* specific activity of 128,620 IU/AU. When the examiner noticed this disclosure late in the prosecution, he rejected the '195 claims with a specific activity limitation of "at least 120,000" as anticipated by the Miyake et al. disclosure. It was only after the "at least 120,000" claims were cancelled that GI submitted the "at least about 160,000" claim language.

The court found the "addition of the word 'about' seems to constitute an effort to recapture ... a mean activity somewhere between 120,000, which the patent examiner found was anticipated by the prior art, and [the] 160,000 IU/AU" claims which were previously allowed. Because "the term 'about' 160,000 gives no hint as to which mean value between the Miyake et al. value of 128,620 and the mean specific activity level of 160,000 constitutes infringement," the court held the "at least about" claims to be invalid for indefiniteness. 13 USPQ2d at 1787-88. This holding was further supported by the fact that nothing in the specification, prosecution history, or prior art provides any

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indication as to what range of specific activity is covered by the term "about," and by the fact that no expert testified as to a definite meaning for the term in the context of the prior art. In his testimony, Fritsch tried to define "about" 160,000, but he could only say that while "somewhere between 155[,000] might fit within that number," he had not "given a lot of direct considerations to that...."

[11] When the meaning of claims is in doubt, especially when, as is the case here, there is close prior art, they are properly declared invalid. *Standard Oil Co. v. American Cyanamid Co.*, 774 F.2d 448, 453, 227 USPQ 293, 297 (Fed. Cir. 1985). We therefore affirm the district court's determination on this issue. We also note that, in view of our reversal of the district court's holding that claims 1 and 3 are valid, it is clear that claims 4 and 6 would also be invalid without the "about" limitation. In arriving at this conclusion, we caution that our holding that the term "about" renders indefinite claims 4 and 6 should not be understood as ruling out any and all uses of this term in patent claims. It may be acceptable in appropriate fact situations, e.g., *W.L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1557, 220 USPQ 303, 316 (Fed. Cir. 1983) ("use of 'stretching ... at a rate exceeding about 10% per second' in the claims is not indefinite"), even though it is not here.

C. Inequitable Conduct

The district court concluded that GI did not engage in inequitable conduct with respect to the '195 patent. Amgen challenges this holding, asserting, *inter alia*, that GI displayed an intent to mislead by withholding data showing *in vivo* specific activity of homogenous uEPO and withholding information on the range of error in EPO bioassays. It is fundamental that to establish inequitable conduct, an intent to deceive is required. *RCA Corp. v. Data General Corp.*, 887 F.2d 1056, 1065, 12 USPQ2d 1449, 1456-57 (Fed. Cir. 1989). A finding of an intent to deceive may

follow from an assessment of materiality, knowledge, and surrounding circumstances, including evidence of good faith. *Kingsdown Medical Consultants Ltd. v. Hollister Inc.*, 863 F.2d 867, 876, 9 USPQ2d 1384, 1392 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1067 (1989). The district court found no such intent, stating: the record is devoid of any evidence that would establish deliberate knowing withholdings of any kind by Dr. Hewick or GI. Dr. Hewick was a credible witness who spoke carefully and candidly about his work ... There is no evidence that Dr. Hewick withheld any information he believed was material to the patent examiner. *Amgen*, 13 USPQ2d at 1791. There is no clear error in this finding. Amgen raises no inequitable conduct issues that were not fully considered by the district court. We have reviewed the record and find no abuse of discretion on the part of the district court. This is also not an exceptional case.

III. OTHER ISSUES

In view of our conclusion that the district court erred as a matter of law in holding that claims 1 and 3 of the '195 patent are not invalid, we vacate the district court's holdings relating to infringement of those claims. We have considered the other arguments by counsel on both sides and find them to be without merit.

CONCLUSION

We conclude that the district court did not err in its findings that claims 2, 4, and 6 of the '008 patent are valid and enforceable and have been infringed by GI, and that claims 7, 8, 23-27, and 29 of the '008 patent are invalid; we therefore affirm the judgment of the court regarding the '008 patent. Because we conclude that claims 1, 3, 4, and 6 of the '195 patent are invalid, we affirm the judgment concerning claims 4 and 6 and reverse the judgment concerning claims 1 and 3.

COSTS

Each party shall bear its own costs.

AFFIRMED-IN-PART, REVERSED-IN-PART, VACATED-IN-PART

Footnotes

Footnote 1. The district court, in a detailed opinion, fully sets out the scientific and historical background relating to the patents at issue. *See Amgen*, 13 USPQ2d at 1741-58. Familiarity with that opinion is presumed.

Footnote 2. Amgen subsequently filed a complaint with the United States International Trade Commission alleging that Chugai's importation of rEPO, manufactured in Japan using genetically engineered host cells, violated Section 337 of the Tariff Act of 1930 (19 U.S.C. §§1337, 1337a). The Commission entered an order terminating the investigation for lack of subject matter jurisdiction. This court vacated and remanded, holding that the Commission should have treated the complaint on the merits and not on jurisdictional grounds, and that the claims of Amgen's patent did not cover a process for producing rEPO. *Amgen, Inc. v. United States Int'l Trade Comm'n*, 902 F.2d 1532, 14 USPQ2d 1734 (Fed. Cir. 1990).

Footnote 3. We note that both the district court and the parties have focused on the obviousness of a process for making the EPO gene, despite the fact that it is products (genes and host cells) that are claimed in the patent, not processes. We have directed our attention accordingly, and do not consider independently whether the products would have been obvious aside from the alleged obviousness of a method of making them.

Footnote 4. At this point, some explanation of the involved technology may be useful, consistent with that expressed in the district court opinion. DNA consists of two complementary strands of nucleotides, which include the four basic compounds adenine(A), guanine(G), cytosine(C), and thymine(T), oriented so that bases from one

strand weakly bond to the bases of the opposite strand. A bonds with T, and G bonds with C to form complementary base pairs. This bonding process is called hybridization and results in the formation of a stable duplex molecule. The structure also includes 5-carbon sugar moieties with phosphate groups.

The genetic code for a particular protein depends upon sequential groupings of three nucleotides, called codons. Each codon codes for a particular amino acid. Since there are four nucleotide bases and three bases per codon, there are 64 (4x4x4) possible codons. Because there are only 20 natural amino acids, most amino acids are specified by more than one codon. This is referred to as a "redundancy" or "degeneracy" in the genetic code, a fact that complicates and renders more difficult the techniques of recombinant DNA.

In order to prepare a protein using recombinant DNA technology, the gene for the protein must first be isolated from a cell's total DNA by screening a library of that cell's DNA. The DNA library is screened by use of a probe, a synthetic radiolabelled nucleic acid sequence which can be used to detect and isolate complementary base sequences by hybridization. To design a probe when the gene has not yet been isolated, a scientist must know the amino acid sequence, or a portion thereof, of the protein of interest. Because some amino acids have several possible codons and the researcher cannot know which of the possible codons will actually code for an amino acid, he or she may decide to design a set of probes that covers all possible codons for each amino acid comprising the protein, known as a "fully-degenerate" set of probes. A library to be screened can be a genomic library (gDNA), which contains a set of all the DNA sequences found in an organism's cells or a complementary DNA (cDNA) library, which is much smaller and less complex than a gDNA library, and is used frequently when the tissue source for a given gene is known.

Footnote 5. Defendants assert that all the claims should be invalid for failure to disclose the best mode. We perceive that the best mode issue only relates to the host cell claims, 4, 6, 23-27, and 29. Absent inequitable conduct, a best mode defense only affects those claims covering subject matter the practice of which has not been disclosed in compliance with the best mode requirement. *See Northern Telecom, Inc. v. Datapoint Corp.*, 908 F.2d 931, 940, 15 USPQ2d 1321, 1328 (Fed. Cir.), *cert. denied*, — U.S. —, 111 S.Ct. 296 (1990).

Footnote 6. In its opinion, the district court stated that "the best way to express EPO was from mammalian cells ... and that a cell line derived from 11 possible clones from the CHO B11, 3,.1 cell strain was to be used for Amgen's master working cell bank, which was expected to be started on November 26, 1984." 13 USPQ2d at 1772. At another point, the court stated that Amgen "did disclose the best mode in Example 10 of the invention, when it described the production rates of the 100 nanomolar-amplified cells (the B11 3,.1 cell strain) and one micromolar-treated cells." *Id.*

Footnote 7. *See also* 53 *Fed. Reg.* 39420, 39425 (Oct. 6, 1989) (comment *re* "deposit [to] satisfy the best mode requirement"); 52 *Fed. Reg.* 34080, 34080 and 34084 (Sept. 8, 1987) (deposit may be required to satisfy enablement, best mode, or distinct claim requirements of §112).

Footnote 8. *Cf. Hormone Research Foundation, Inc. v. Genentech, Inc.*, 904 F.2d 1558, 15 USPQ2d 1039 (Fed. Cir. 1990). In *Hormone Research*, this court, in a remand, directed the district court to consider the effect of *United States Steel Corp. v. Phillips Petroleum Co.*, 865 F.2d 1247, 8 USPQ2d 1461 (Fed. Cir. 1989) and *In re Hogan*, 559 F.2d 595, 194 USPQ 527 (CCPA 1977) on *Fisher* in its enablement analysis. The facts of our case are distinguishable from those in *Hormone Research*, *United States Steel*, and *Hogan*.

Footnote 9. The probes designated "EpV" were from EPO amino acid sequence region 46-52.

Footnote 10. The potency of EPO in the '195 patent is stated as its specific activity, expressed as a ratio of International Units (which measures the ability of EPO to cause formation of red blood cells) per absorbance unit (the amount of light absorbed by a sample of EPO measured by a spectrophotometer at a given wavelength, 280 nanometers), *i.e.*, IU/AU.

Footnote 11. Defendants provided no evidence that faulty purification procedures or other missteps caused its failure to obtain 160,000 IU/AU *in vivo* material as claimed in the '195 patent.

Footnote 12. The court quoted Chugai to the effect that the *in vivo* activity of uEPO is 65 percent that of rEPO.

Footnote 13. Chugai's sample reported to the Food and Drug Administration was not purified by the disclosed process.

- End of Case -

FULL TEXT OF CASES (USPQ2D)
All Other Cases

**Life Technologies Inc. v. Clontech Laboratories Inc., 56 USPQ2d 1186
(CA FC 2000)**

56 USPQ2D 1186
Life Technologies Inc. v. Clontech Laboratories Inc.
U.S. Court of Appeals Federal Circuit

No. 99-1550
Decided September 21, 2000

Life Technologies Inc. v. Clontech Laboratories Inc.

Headnotes

PATENTS

[1] Infringement — Defenses — Fraud or unclean hands (§120.1111)

Showing that patent applicant was responsible for inequitable conduct during prosecution of patent application, which renders patent unenforceable, requires clear and convincing evidence that false, material information was submitted, material facts affirmatively misrepresented, or material information not disclosed, with intent to deceive the U.S. Patent and Trademark Office; if threshold is satisfied, trial court will then balance materiality and intent to determine applicant's culpability.

[2] Practice and procedure in Patent and Trademark Office — Prosecution — Duty of candor — Materiality (§110.0903.04)

Infringement — Defenses — Fraud or unclean hands (§120.1111)

Federal district court erred in finding that inventors of patent in suit engaged in inequitable conduct by disclosing prior art article to U.S. Patent and Trademark Office, but failing to disclose information concerning manner in which they used that reference, since information regarding subjective motivations of inventors is not material, and path that leads inventor to invention is expressly made irrelevant to patentability by 35 U.S.C. §103(a), and since inventors' reliance on article in question, and motivations that they derived from it, thus have no bearing on issue of patentability; in present case, only inquiry is whether teachings of article, in combination with other relevant prior art, would have rendered claimed invention obvious.

[3] Infringement — Defenses — Fraud or unclean hands (§120.1111)

Inventors of patent in suit did not make material misrepresentations regarding prior art article by arguing that article, which was contrary to established teachings and was based on relatively new technique, did not promise reasonable expectation of success at time invention was made and therefore did not render their claimed invention obvious, since inventors merely advocated particular interpretation of teachings of article, which patent examiner was free to accept or reject, and since inventors' argument is not contradicted by fact that they successfully used article to achieve desired result, in that using inventors' success as evidence that success would have been expected is impermissible use of hindsight.

[4] Infringement — Defenses — Fraud or unclean hands (§120.1111)

Inventors of patent in suit did not withhold material information concerning work of rival researcher, since inventors were in possession

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of only very limited information regarding researcher's work, and at most could have disclosed only that researcher claimed to have achieved results similar to those of claimed invention, and presented his results at conference which neither inventor attended, since this information lacks specificity and definiteness required to support patentability rejection under 35 U.S.C. §102(g), since inventors' incomplete knowledge regarding researcher would not have been material to reasonable patent examiner, and since inventors did not obtain any material regarding researcher's work based on his presentation.

Particular patents — Chemical — Genetic cloning

5,244,797, Kotewicz and Gerard, cloned genes encoding reverse transcriptase lacking RNase H activity, judgment of unenforceability reversed.

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5,668,005, Kotewicz and Gerard, cloned genes encoding reverse transcriptase lacking RNase H activity, judgment of unenforceability reversed.

Case History and Disposition

Appeal from the U.S. District Court for the District of Maryland, Williams, J.

Action by Life Technologies Inc. against Clontech Laboratories Inc. for patent infringement. Following bench trial, federal district court held plaintiff's patents unenforceable on ground of inequitable conduct, and plaintiff appealed. Reversed and remanded.

Attorneys:

Robert J. Koch, of Fulbright & Jaworski, Washington, D.C.; Scott H. Blackman, of Lyon & Lyon, Washington; Steven M. Bauer, of Testa, Hurwitz & Thibault, Boston, Mass.; Alan W. Hammond, of Life Technologies Inc., for plaintiff-appellant.

Marc R. Labgold, of Piper, Marbury, Rudnick & Wolfe, Washington; Catherine B. Richardson, Sharon E. Crane, and Kevin M. Bell, of Long, Aldridge & Norman, Washington, for defendant-appellee.

Judge:

Before Michel, Bryson, and Gajarsa, circuit judges.

Opinion Text

Opinion By:

Gajarsa, J.

Life Technologies, Inc. ("LTI") appeals the judgment of the United States District Court for the District of Maryland, entered after a bench trial, in which the court held that LTI's U.S. Patents Nos. 5,244,797 ("the '797 patent") and 5,668,005 ("the '005 patent") were unenforceable on the ground of inequitable conduct. Because the court premised this determination on clearly erroneous findings of fact, we reverse and remand.

BACKGROUND

Reverse transcriptase ("RT") is a naturally occurring enzyme that exhibits DNA polymerase activity. DNA polymerase activity enables the RT enzyme to utilize a messenger RNA ("mRNA") molecule as a template to synthesize a complementary strand of DNA ("cDNA"). This reaction results in a DNA/RNA hybrid molecule. In addition to DNA polymerase activity, naturally occurring RT, known as "wild-type"RT, also exhibits RNase H activity. RNase H activity degrades the original mRNA template as the cDNA molecule is made. RNase H activity is undesirable because this degradation of the mRNA template negatively affects the ability and efficiency of the RT to make cDNA.

Beginning in the early 1980's, the inventors of the '797 and '005 patents, Drs. Michael Kotewicz and Gary Gerard, sought to develop a genetically engineered RT enzyme that exhibited DNA polymerase activity but did not substantially exhibit RNase H activity. Kotewicz and Gerard faced several difficulties in developing this enzyme.

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First, it was unknown at the time where on the RT molecule the DNA polymerase and RNase H activities resided. Further, it was unknown whether the RNase H activity could be selectively removed to produce an improved mutant RT enzyme that retained DNA polymerase activity. The inventors spent several years unsuccessfully attempting to locate and delete the RNase H activity from the RT molecule.

The breakthrough for the inventors came in 1986 with the publication of M. S. Johnson et al., *Computer Analysis of Retroviral Pol Genes: Assignment of Enzymatic Functions to Specific Sequences and Homologies with Nonviral Enzymes*, 83 Proceedings of the Nat'l. Acad. of Sci. 7648 (1986) ("the Johnson article"). The Johnson article reported comparisons made between amino sequences of certain RT molecules and the sequence of the ribonuclease enzyme from *E. coli*, which exhibits RNase H activity but not DNA polymerase activity. The findings of Johnson suggested to the inventors that the RNase H activity of the RT enzyme resided at the carboxyl

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terminal end of the molecule. The inventors, however, were skeptical of Johnson's results because the literature existing at the time suggested that the location of the RNase H activity was at the front end of the RT molecule. Additionally, the Johnson article was suspect because it utilized computer comparisons of amino acid sequences, rather than experimental data. Such computer comparisons were fairly new in the art at the time. Thus, in order to "exclude the possibility" that Johnson was correct, Kotewicz and Gerard decided to conduct experiments at the carboxyl terminal end of the RT enzyme. Contrary to expectations, these experiments were successful and, by December 1986, the inventors had created a mutant RT enzyme that lacked RNase H activity but retained DNA polymerase activity.

A few months after confirming their discovery, Kotewicz and Gerard learned that another researcher, Dr. Stephen Goff, was working to develop an engineered RT enzyme. On April 15, 1987, Kotewicz spoke on the telephone with Goff, a researcher at Columbia University. During this conversation, Goff stated that he had developed "oligonucleotide insertion mutations that reduce RNase H in cloned [RT]." However, there is no indication in the record that Goff revealed any further details concerning his work to Kotewicz during this conversation. Additionally, during the summer of 1987, Gerard learned that Goff had presented his RT research at Stanford University. Although neither Gerard nor Kotewicz attended this presentation, they surmised, based on conversations with colleagues, that Goff demonstrated "similar results" as the inventors. Based on this information, Kotewicz and Gerard urged LTI to allow them to publish their results as quickly as possible, under the assumption that Goff would soon publish similar work. They also submitted forms to management at LTI that initiated the process for preparing a patent application for their engineered RT enzyme.

In January 1988, Kotewicz and Gerard filed the parent application from which the '797 and '005 patents ultimately issued. In general, the application claimed an engineered RT enzyme that exhibited DNA polymerase activity but did not exhibit substantial RNase H activity. As part of the duty of disclosure under 37 C.F.R. §1.56, the inventors disclosed to the Patent and Trademark Office ("PTO") numerous prior art references, including the Johnson article. However, the inventors did not reveal their knowledge of Goff's work because their patent attorney indicated that such limited and incomplete information would not be material.

During the prosecution leading to the '797 patent, the Johnson article took on particular importance for the Examiner. Several times, the Examiner rejected the inventors' claims as obvious over Johnson, often in combination with other prior art that described the RT gene sequence. According to the Examiner, because Johnson taught that the RNase H activity was located at the carboxyl terminal end, and because there was a strong motivation in the art to eliminate such activity, the claimed invention would have been obvious. In response to these rejections, the inventors argued that, at the time of the invention, there would have been no reasonable

expectation that the application of Johnson's results would successfully lead to the deletion of RNase H activity. This was because the teachings of Johnson were contrary to teachings in the prior art which suggested that "something more was necessary" than a deletion at the carboxyl terminal end to eliminate RNase H activity. Thus, the inventors contended that the claimed invention would not have been obvious over Johnson. The Examiner was persuaded by these arguments, and the '797 patent issued on September 19, 1993. At no time during this prosecution did the inventors reveal to the Examiner that the Johnson article played a key role in their development of the claimed invention.

Shortly after the issuance of the '797 patent, the inventors filed the continuation application that eventually resulted in the issuance of the '005 patent. During the prosecution of this application, the inventors revealed their knowledge of Goff's work. The Examiner allowed the application to issue over the newly revealed information regarding Goff, stating that the new information had "no bearing on ... the instant application."

In December 1996, LTI sued Clontech Laboratories, Inc. ("Clontech") for infringement of the '797 and '005 patents. In response, Clontech asserted various affirmative defenses, including an allegation that the patents should be held unenforceable due to inequitable conduct. A bench trial on the inequitable conduct issue ensued. After the trial, the court found that the inventors withheld material

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information regarding the motivations that they derived from the Johnson article and their reliance on it in reaching their invention. The court also found that the inventors made affirmative material misrepresentations during prosecution regarding the Johnson article. Further, the court found that the inventors' knowledge of Goff's work was material and should have been revealed to the PTO during the prosecution of the '797 patent. Finally, the court determined that these actions were done with the intent to deceive the PTO. As a result, the court held that both the '797 and '005 patent were unenforceable on the ground of inequitable conduct. This appeal followed.

DISCUSSION

Applicants for patents, including their legal representatives, have the duty to prosecute patent applications in the PTO with candor, good faith, and honesty. See *Molins PLC v. Textron, Inc.*, 48 F.3d 1172, 1178, 33 USPQ2d 1823, 1826 (Fed. Cir. 1995); 37 C.F.R. §1.56 (1999). When this duty is breached, the applicant has committed inequitable conduct. A determination of inequitable conduct during the prosecution of a patent application renders the subsequently issued patent unenforceable. See *LaBounty Mfg., Inc. v. United States Int'l Trade Comm'n*, 958 F.2d 1066, 1070, 22 USPQ2d 1025, 1028 (Fed. Cir. 1992).

[1] Inequitable conduct can consist of affirmative misrepresentations of material fact, submission of false material information, or the failure to disclose known material information during the prosecution of a patent, coupled with the intent to deceive the PTO. See *Molins*, 48 F.3d at 1178, 33 USPQ2d at 1826. Materiality and intent to deceive are distinct factual inquiries, and each must be shown by clear and convincing evidence. See *Elk Corp. v. GAF Bldg. Materials Corp.* 168 F.3d 28, 30, 49 USPQ2d 1853, 1855 (Fed. Cir. 1999); *Molins*, 48 F.3d at 1178, 33 USPQ2d at 1826 ("Materiality does not presume intent, which is a separate and essential component of inequitable conduct." (quoting *Allen Organ Co. v. Kimball Int'l, Inc.*, 839 F.2d 1556, 1567, 5 USPQ2d 1769, 1778 (Fed. Cir. 1988))). Once these threshold levels of materiality and intent are satisfied, the ultimate determination of inequitable conduct is within the discretion of the trial court, which must make the equitable judgment concerning whether the applicant's conduct is so culpable that the patent should not be enforced. See *Kingsdown Med. Consultants, Ltd. v. Hollister, Inc.*, 863 F.2d 867, 876, 9 USPQ2d 1384, 1392 (Fed. Cir. 1988) (en banc); *LaBounty*, 958 F.2d at 1070, 22 USPQ2d at

1028. In making this determination, the court must conduct a balancing test between the levels of materiality and intent, with a greater showing of one factor allowing a lesser showing of the other. *See Critikon, Inc. v. Becton Dickinson Vascular Access, Inc.*, 120 F.3d 1253, 1256, 43 USPQ2d 1666, 1668(Fed. Cir. 1997).

Because the determination of inequitable conduct is ultimately committed to the discretion of the district court, we review its determination for abuse of discretion. *See Kingsdown*, 863 F.2d at 876, 9 USPQ at 1392. We must reverse a discretionary ruling of the district court when it is premised upon clearly erroneous findings of fact or on a misapplication or misinterpretation of applicable law, or evidences a serious error of judgment. *See id.* Thus, the district court's factual findings on the issues of materiality and intent are reviewed for clear error, and will not be reversed unless this court has a "definite and firm conviction that a mistake has been committed." *Elk Corp.*, 168 F.3d at 31, 49 USPQ2d at 1855 (quoting *Molins*, 48 F.3d at 1178, 33 USPQ2d at 1827).

The district court determined that the inventors committed inequitable conduct based on three actions during the prosecution of the '797 patent—withholding information regarding their "reliance" on the Johnson article, making material misrepresentations regarding the Johnson article, and withholding information regarding Goff's work. We will address each of these actions in turn.

1. Withholding Information Regarding the Johnson Article

[2] As described above, the Johnson article was extremely influential in leading the inventors to their engineered RT enzymes. Realizing its materiality to the patentability of the claimed invention, the inventors revealed the Johnson article to the PTO during prosecution and even discussed it in the written description section of the patent application. The district court, however, determined that merely disclosing the reference itself was insufficient to comply with the duty of candor. Relying heavily on the testimony of former PTO Commissioner Harry Manbeck, the court found

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that the inventors should have also disclosed their "reliance" on Johnson, and how it motivated them to conduct the experiments that eventually led to the invention. Thus, the district court determined that the manner in which the inventors used a disclosed prior art reference is material information and must be revealed to the PTO. Because this factual finding was premised upon a misapprehension of the legal standards of patentability, it is clearly erroneous.

Information is material when there is a substantial likelihood that a reasonable Examiner would have considered the information important in deciding whether to allow the application to issue as a patent. *See Molins*, 48 F.3d at 1179, 33 USPQ2d at 1827. Because patentability is assessed from the perspective of the hypothetical person of ordinary skill in the art, information regarding the subjective motivations of inventors is not material. *See Standard Oil Co. v. American Cyanamid Co.*, 774 F.2d 448, 454, 227 USPQ 293, 297(Fed. Cir. 1985) (noting that the person of ordinary skill in the art is an objective legal construct presumed to think along conventional lines without undertaking to innovate, whether by systematic research or by extraordinary insights). As stated by this court in *Standard Oil*:

Inventors, as a class, according to the concepts underlying the Constitution and the statutes that have created the patent system, possess something—call it what you will—which sets them apart from the workers of ordinary skill, and one should not go about determining obviousness under §103 by inquiring into what patentees ... would have known or would likely have done, faced with the revelations of references.

Id. Furthermore, the path that leads an inventor to the invention is expressly made irrelevant to patentability by

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statute. See 35 U.S.C. §103(a) ("Patentability shall not be negated by the manner in which the invention was made."). Thus, the inventors' reliance on the Johnson article and the motivations that they derived from it have no bearing on the issue of patentability. It does not matter whether the inventors reached their invention after an exhaustive study of the prior art, or developed their RT enzymes in complete isolation. The only inquiry is whether the teachings of the Johnson article, in combination with other relevant prior art, would have rendered the claimed invention obvious to one of ordinary skill in the art; this inquiry, as a matter of law, is independent of the motivations that led the inventors to the claimed invention. Therefore, the district court clearly erred by finding that the inventors' reliance on Johnson was material.

2. Material Misrepresentations Regarding the Johnson Article

The district court also found that the inventors made affirmative misrepresentations regarding the Johnson article. During the prosecution of the '797 patent, the inventors were faced with rejections based on Johnson, which taught the location of the RNase H activity, in combination with other prior art references that described the RT enzyme itself. In response to these rejections, the inventors argued that this combination did not render the claimed invention obvious because at the time the invention was made, one of ordinary skill in the art would have thought that "something more was necessary" than a deletion at the carboxyl terminal end to eliminate RNase H activity. Thus, they argued, there would have been no reasonable expectation of success in applying Johnson's teachings. The court determined that this was a misrepresentation because the inventors later admitted that the Johnson article correctly taught the location of RNase H activity and led them directly to the claimed invention. In effect, the court determined that inventors could not truthfully argue lack of reasonable expectation of success as a basis for non-obviousness when they successfully used the prior art reference at issue. The court clearly erred in this determination.

[3] It is axiomatic that a claimed invention is not obvious solely because it is composed of elements that are all individually found in the prior art. See, e.g., *In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457 (Fed. Cir. 1998). Thus, even though the location of the RNase H activity is taught in the Johnson article, the claimed invention of the '797 patent is not *per se* obvious. For the Johnson article to render the claimed invention obvious, there must have been, at the time the invention was made, a reasonable expectation of success in applying Johnson's teachings. See *Micro Chem., Inc. v. Great Plains Chem. Co.*, 103 F.3d 1538, 1547, 41 USPQ2d 1238, 1245 (Fed. Cir. 1997); *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988). Because the Johnson article was contrary

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to established teachings and was based on a relatively new technique, the inventors argued that a reasonable expectation of success was lacking. This argument is simply not a misrepresentation.

First, in making the argument, the inventors merely advocated a particular interpretation of the teachings of the Johnson article and the level of skill in the art, which the Examiner was free to accept or reject. This argument did not contain any factual assertions that could give rise to a finding of misrepresentation. See, e.g., *Akzo N.V. v. United States Int'l Trade Comm'n*, 808 F.2d 1471, 1482, 1 USPQ2d 1241, 1247 (Fed. Cir. 1986) (holding that an argument for distinguishing prior art, even though favorable to the applicant's position, was not a material misrepresentation because the Examiner could reach his/her own conclusions regarding the prior art). Furthermore, the inventors' argument is not contradicted by the fact that they successfully used the Johnson article to achieve the desired result. Reasonable expectation of success is assessed from the perspective of the person of ordinary skill in the art. See *Micro Chem.*, 103 F.3d at 1547, 41 USPQ2d at 1245. That the inventors were ultimately successful is irrelevant

to whether one of ordinary skill in the art, at the time the invention was made, would have reasonably expected success. *See Standard Oil*, 774 F.2d at 454, 227 USPQ at 297. The court's finding to the contrary represents impermissible use of hindsight—using the inventors' success as evidence that the success would have been expected. *See In re Kotzab*, 217 F.3d 1365, 1369, 55 USPQ2d 1313, 1316 (Fed. Cir. 2000) (noting the importance of casting the mind back to the time of the invention to avoid the “insidious effect of a hindsight syndrome wherein that which only the invention taught is used against its teacher”). In short, the inventors' non-obviousness arguments were not affirmative misrepresentations and cannot give rise to a determination of inequitable conduct. Thus, the district court committed clear error.

3. Withholding Knowledge of Goff's Work

[4] Finally, the district court determined that the inventors had withheld material information regarding Goff's work. Specifically, the court found that the inventors should have revealed that Goff claimed to have developed “oligonucleotide insertion mutations that reduce[d] RNase H in cloned [RT],” and had presented these results at a conference during the summer of 1987. The court clearly erred in finding that this information was material.

As described above, information is material when there is a substantial likelihood that a reasonable Examiner would have considered the information important in deciding whether to allow the application to issue as a patent. *See Molins*, 48 F.3d at 1179, 33 USPQ2d at 1827. The inventors were in possession of only very limited information regarding Goff's work. At most, the inventors could have disclosed to the PTO that a rival researcher claimed to have reduced RNase H activity in cloned RT and had presented his results at a conference, which neither of the inventors attended. Clontech contends that this information was material because it indicated that Goff was a prior inventor. However, this information lacks the specificity and definiteness required to support a patentability rejection under 35 U.S.C. §102(g).

To establish prior inventorship, one must show either a prior reduction to practice of the invention or a prior conception followed by reasonable diligence in reducing the invention to practice. *See Eaton v. Evans*, 204 F.3d 1094, 1097, 53 USPQ2d 1696, 1698 (Fed. Cir. 2000). While the inventors knew that Goff had potentially achieved a reduction in RNase H activity in RT enzymes, the inventors were unaware of when these results were achieved, how they were achieved, the degree to which the RNase H activity was reduced, or whether DNA polymerase activity was retained. Knowledge of these details would have been required for the Examiner to consider whether Goff was a prior inventor. Because the inventors lacked such crucial information, the incomplete knowledge that they did have regarding Goff would not have been material to a reasonable Examiner. *See Hartness Int'l, Inc. v. Simplimatic Eng'g Co.*, 819 F.2d 1100, 1107, 2 USPQ2d 1826, 1831 (Fed. Cir. 1987) (holding that inequitable conduct had not been established when the patentee had only “heard of” a third-party usage). This conclusion is supported by the fact that when the substance of the Goff telephone conversation was revealed to the Examiner during the prosecution of the '005 patent, the Examiner stated that the information had “no bearing on the patents issued or the instant application.” Such a strong statement regarding the Goff information is highly probative of its immateriality.

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See Molins, 48 F.3d at 1179, 33 USPQ2d at 1827 (“[T]he result of a PTO proceeding that assesses patentability in light of information not originally disclosed can be of strong probative value in determining whether the undisclosed information was material.”).

Moreover, the inventors did not obtain any material information regarding Goff's work based on his presentation at Stanford. The inventors neither attended this presentation, nor were provided with an abstract of

the presentation. They had heard from colleagues that Goff presented results similar to theirs, but the record does not show that the inventors learned any additional details regarding Goff's work. Thus, the inventors could not have revealed anything to the Examiner regarding this presentation beyond conjecture and a vague report that "similar results" were presented. Such incomplete information would be singularly unhelpful to the Examiner in determining whether the invention was patentable, and consequently, would not be material.

CONCLUSION

The district court clearly erred in finding that the inventors withheld material information and made affirmative misrepresentations during the prosecution of the patents at issue. Therefore, the court's determination that the inventors engaged in inequitable conduct is reversed, and this case is remanded for further proceedings.

COSTS

Each party shall bear its own costs.

REVERSED AND REMANDED.

- End of Case -

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